ANALYSIS OF THE MRNA-BINDING PROTEIN NAB2 DURING THE NUCLEAR EXPORT MECHANISM

By

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To my dad

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LIST OR ABBREVIATIONS

Δ	Null
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BPB	Bromophenyl blue
CBC	Cap binding complex
CC1	Commitment complex
CWI	Cell wall integrity
DAPI	4'-6-Diamidino-2-phenylindole
DDT	Dithiothreitol
DEAD	Aspartic acid - glutamic acid - alanine - aspartic acid
DIGE	Differential gel electrophoresis
DM	Myotonic dystrophy
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
DMPK	Myotonic dystrophy protein kinase
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EJC	Exon junction complex

ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FG	Phenylalanine - glycine
FMRP	Fragile X mental retardation protein
FxFG	Phenylalanine - any amino acid - phenylalanine - glycine
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GLFG	Glycine - leucine - phenylalanine - glycine
GTP	Guanosine triphosphate
hnRNP	Heterogeneous nuclear ribonucleoproteins
HSP	Heat shock protein
IMAC	Immobilized metal ion-affinity chromatography
FG	Phenylalanine glyine
GFP	Green fluorescent protein
GO	Gene ontology
GST	Glutathione S-transferase
h	Human
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IP_6	Inositol hexaphosphate

IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	KiloDalton
LC/MS	Liquid chromatography/mass spectrometry
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	MAP/ERK kinase
MES	2-(N-morpholino)ethanesulfonic acid
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoparticle
MS	Mass spectrometry
MudPIT	Multidimensional protein identification technology
NPC	Nuclear pore complex
Nup	Nucleoporin
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
poly(A)$^+$	Polyadenylation
PrA	Protein A
psi	Pounds per square inch
PVP	Polyvinylpyrrolidone
R	Arginine

RNA	Ribonucleic acid
RT PCR	Reverse transcription polymerase chain reaction
S	Serine
SC	Synthetic complete growth media
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SGD	*Saccharomyces* Genome Database
siRNA	Small interfering ribonucleic acid
SMM	Sulfometuron methyl
TAP	Tandem affinity purification
TREX	Transcription export complex
TREX2	Transcription export complex two
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
UV	Ultraviolet
WT	Wild type
YPD	Yeast extract peptone dextrose

CHAPTER I

INTRODUCTION

Nuclear mRNA export

Eukaryotic gene expression is controlled by multiple mechanisms, and its regulation is central for physiological responses to extracellular and intracellular signals. An essential step in this process involves the movement of mRNA transcripts from the site of synthesis in the nucleus to the cytoplasm, where they can be translated into proteins. The nuclear export of mRNA transcripts can be broken down into distinct stages: first, pre-mRNA is transcribed in the nucleus, where it is processed and packaged into messenger ribonucleoparticle (mRNP) complexes. Second, the mRNP complexes are targeted to and translocate through nuclear pore complexes (NPCs) that are embedded in the nuclear envelope. Third, the mRNP complexes are directionally released into the cytoplasm for translation. Recent work has revealed that there is extensive mechanistic coupling between each of these steps (Kohler and Hurt, 2007). It has also been shown that individual sets of mRNAs can be selectively exported in response to different cellular stress conditions (Izawa et al., 2008). Moreover, it has been shown that perturbations in the factors that are essential for mRNA nuclear export have surprising links to different disease states. In this introduction, I discuss each of these facets of the mRNA nuclear export pathway, detailing the field as it currently stands, and providing a background for which my studies build upon.

This chapter is adapted from Carmody and Wente 2009, Journal of Cell Science 122: 1933-1937.

Early mRNA processing events: Assembly of mRNPs

The formation of an export-competent mRNP complex begins at transcription. During transcriptional elongation, the nascent mRNA transcript is bound by a number of factors, some of which are from the family of heterogeneous nuclear ribonucleoproteins (hnRNPs) (Figure 1, Table1). HnRNPs are highly abundant, nuclear RNA-binding proteins that are essential for various steps in the mRNA lifecycle, including packaging, 3'-end formation, export and translation. There are ~30 different hnRNPs in humans, and ~10 in the budding yeast *Saccharomyces cerevisiae* (Dreyfuss et al., 2002). Interestingly, many individual hnRNP proteins are important for multiple different roles in the mRNA life cycle.

An example of one such protein in *S. cerevisiae* is the SR-like protein Npl3. SR proteins (named for their richness in serine (S) and arginine (R) residues) are a family of mRNA-binding proteins classically described as splicing factors, although many SR proteins have been found to play roles in other processes as well. Npl3 is considered an SR-like protein due to its partial homology to *bona fide* SR proteins in higher eukaryotes. Npl3 is an essential protein in *S. cerevisiae*, which binds mRNA co-transcriptionally, and is exported into the cytoplasm bound to mRNA (Lee et al., 1996). While involved in pre-mRNA splicing (Kress et al., 2008) as its SR-like domain would suggest, Npl3 has also been implicated in a multitude of other functions, including transcription elongation, transcription termination, 3'-end formation, and mRNA export in the nucleus and repression of translation in the cytoplasm (Bucheli and Buratowski, 2005; Dermody et al., 2008; Lee et al., 1996, Windgassen et al., 2005; Wong et al., 2010).

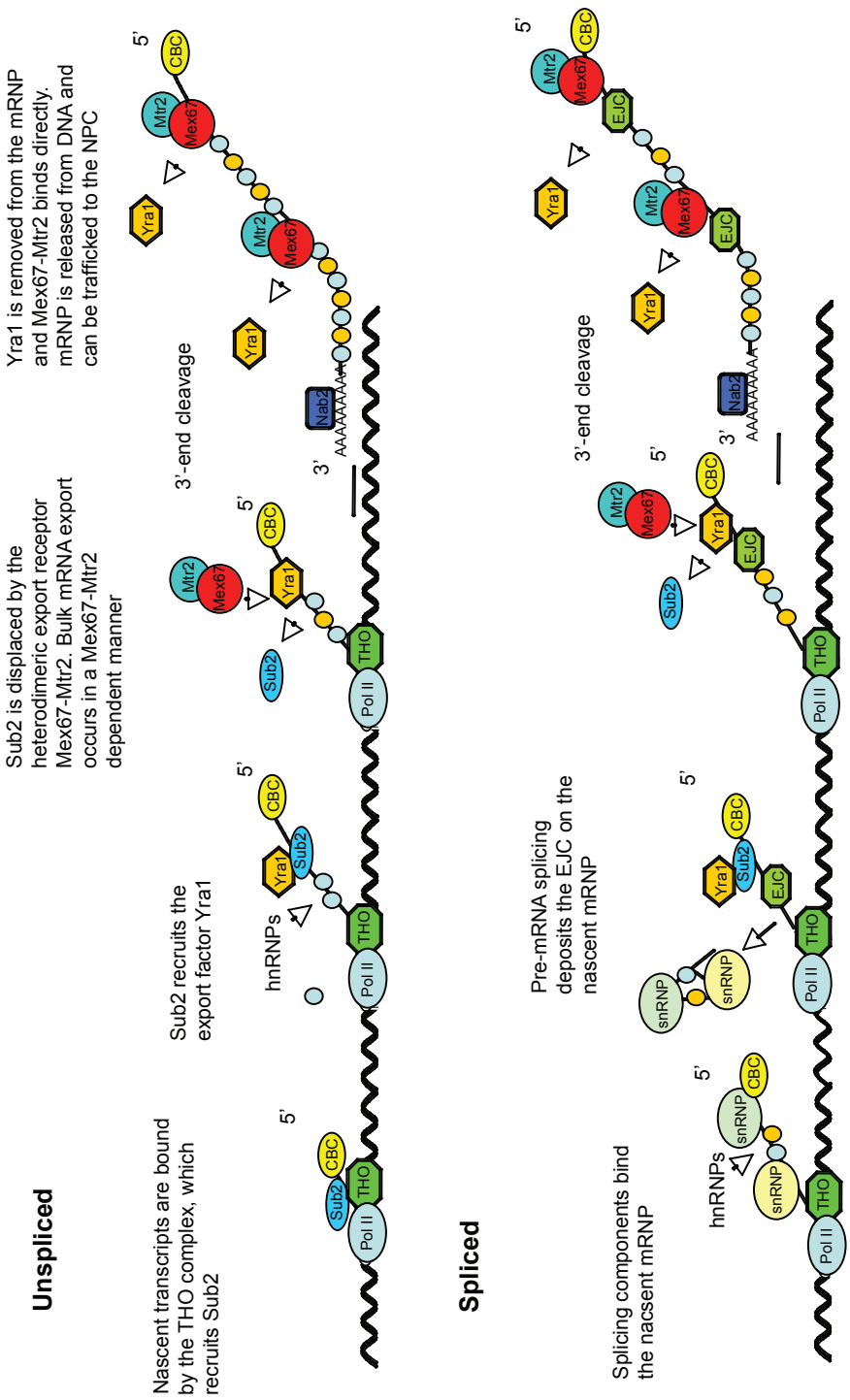

Figure 1: mRNA export mediated by Mex67-Mtr2. Bulk mRNA is exported by the heterodimeric mRNA export receptor Mex67:Mtr2. Maturation steps are described step-by-step in the figure.

Table 1: Factors involved in nuclear mRNA export

S. cerevisiae	Metazoan	Function
Mex67	Nxf1/TAP	mRNA export receptor; bridges mRNP interaction with FG Nups in NPC; forms heterodimer with Nxt1
Mtr2	Nxt1/p15	Forms heterodimer with Nxf1
Sub2	UAP56/HEL	DEAD box protein; member of TREX; incorporated into mRNP via THO; recruits ALY
Yra1	ALY/Ref	Member of TREX; incorporated into mRNP via UAP56; recruits Nxf1-Nxt1 to mRNP
CBC	CBC	Nuclear 5' cap binding complex; comprised of Cbp20 and Cbp80; replaced by eIF complex in the cytoplasm
THO	THO	Protein complex involved in transcription elongation; Comprised of Tho2, Hpr1, Mft1 and Thp2 in *S. cerevisiae*, and hTho2, hHpr1, fSAP79, fSAP35 and fSAP24
TREX	TREX	Transcription export complex; formed from THO association with UAP56, ALY in metazoans and Sub2, Yra1, and Tex1 in *S. cerevisiae*
hnRNPs	hnRNPs	Heterogeneous nuclear ribonucleoproteins; bind mRNA; function in processing events and export
	PABPII	Nuclear poly(A^+) binding protein. No homologue in *S. cerevisiae* (Nab2 functionally similar)
EJC	EJC	Exon junction complex; deposited at site of exon fusion; recruits THO complex in metazoans
Dbp5	Dbp5/DDX19	DEAD box protein; RNA-dependent ATPase; Docks at NPC via Nup214; ADP bound form acts to remodel mRNP proteins (Mex67 and Nab2 in *S. cerevisiae*)
Gle1	hGle1	Docks at NPC via hCG1. In *S. cerevisiae*, binds IP_6 to stimulate Dbp5 ATPase activity
Nup159	Nup214/CAN	Nucleoporin on cytoplasmic face; Dbp5 docking site
Nup42	hCG1	Nucleoporin on cytoplasmic face; Gle1 docking site

Pab1	PABPI	Cytoplasmic poly(A^+) binding protein; mediates interaction between 3' tail, and cap during translation
IP_6	IP_6	Inositol hexakisphosphate; binds Gle1.
Xpo1	CRM1	Karyopherin; protein export receptor; exports mRNA via adaptor proteins (HIV mRNA and subset of endogenous mRNAs)

Another example from *S. cerevisiae* of an hnRNP protein involved in multiple functions in the mRNA lifecycle is Nab2. Nab2 is a poly(A^+) -binding protein, which, similar to the Npl3, is essential, binds mRNA co-transcriptionally and is exported into the cytoplasm while bound to mRNA. Also like Npl3, Nab2 has multiple functions in the lifecycle of an mRNA, playing roles in both polyadenylation and mRNA export (Anderson et al., 1993; Hector et al., 2002). Npl3 and Nab2 are but two examples of hnRNP proteins that are essential in multiple facets of the maturation of their associated mRNA. Many other such examples exist and as such, the discussion of Npl3 and Nab2 is intended only to give a glimpse of the complexity of the roles of hnRNP proteins on their associated mRNAs, and not to be an exhaustive description.

While there are many more hnRNP proteins in humans than there are in *S. cerevisiae*, and relatively minimal sequence conservation within many members of the group, several core functions are conserved. For example, human cells harbor a nuclear poly(A^+)-binding protein, PABP2 (Dreyfuss et al., 2002), and a cytoplasmic poly(A^+)-binding protein, PABP1 (Burd et al., 1991). In *S. cerevisiae,* Pab1 is a homologue of human PABP1 (Caponigro and Parker, 1995) but the nuclear poly(A^+)-binding protein, Nab2, is not structurally similar to human PABP2 (Anderson et al., 1993).

Different hnRNP proteins associate with the mRNP complex at distinct steps in the export pathway. Notably, some hnRNPs harbor nuclear-retention signals and are removed prior to export of the mRNP, whereas others are retained during export and released in the cytoplasm, then shuttle back into the nucleus (Dreyfuss et al., 2002). By associating, and disassociating with the mRNP at different steps in its lifecycle, and being involved in such a wide array of functions, hnRNP proteins have the unique ability to

influence mRNA processing, and thus gene expression, at every point in the mRNA's lifespan, from the early nuclear processes of transcription, splicing, and 3'-end formation, to nuclear export, to cytoplasmic translation and eventually decay.

Key steps in mRNA processing

There are four main processing events that occur during the formation of a mature mRNA transcript: 5' capping, splicing, 3'-end cleavage and polyadenylation (Figure 1). Each of these modifications impacts export in two ways. First, if an mRNA is not properly processed, it will not be exported and instead will be targeted for degradation. Second, the processing events serve as triggers to recruit protein factors that are necessary for export.

The first change that a nascent pre-mRNA transcript undergoes is 5' capping. When a transcript reaches ~20-30 nucleotides in length, a 7-methylguanosine cap is added to the 5' end, which protects the nascent pre-mRNA from degradation (Shatkin and Manley, 2000). In microinjected *Xenopus laevis* oocytes, uncapped mRNA is either poorly exported from the nucleus, or not exported at all (Cheng et al., 2006). The 5' cap is bound by the cap-binding complex (CBC) (Izaurralde et al., 1995). Next, a transcript undergoes splicing and a set of proteins is simultaneously deposited at the site of exon fusion. These proteins are defined as the exon-junction complex (EJC). Capping and splicing are both important for the recruitment of the transcription-export (TREX) complex (Cheng et al., 2006; Masuda et al., 2005). The TREX complex is highly conserved and is essential for mRNA export. It consists of the THO complex (which is made up of several components; see table**)** and a set of export factors (Sub2 (an ATP-

dependent DEAD-box RNA helicase),Yra1 and Tex1 in *S. cerevisiae*; UAP56, a Sub2 homologue (also called HEL) and ALY, a Yra1 homologue (also called REF) in human cells) (Masuda et al., 2005; Piruat and Aguilera, 1998; Strasser et al., 2002) (Figure 1, Table1). In *S. cerevisiae,* the THO complex associates with the nascent mRNA during transcription, and participates in both transcription elongation and mRNA export. Yeast strains that have mutations in any of the genes encoding the four THO-complex members have defects in mRNA export, transcription elongation and show an accumulation of transcripts in foci at or near their sites of transcription (Jimeno et al., 2002; Strasser et al., 2002). Once it is associated with the mRNA, the THO complex then recruits the remaining TREX-complex components (Strasser et al., 2002). Interestingly, in higher eukaryotes, the TREX complex is poorly recruited to transcripts that lack either the 5' cap or the exon junction complex (EJC), indicating that its mechanism of recruitment is linked to splicing and/or capping and not to transcription (Cheng et al., 2006; Zhou et al., 2000).

The final pre-mRNA processing events are 3'-end cleavage and polyadenylation. A polyadenylation site is recognized in the 3'-untranslated region (UTR), resulting in pre-mRNA cleavage immediately downstream. The poly(A^+) tail is added by a poly(A^+) polymerase and bound by poly(A^+)-binding protein (Proudfoot, 2004). Notably, studies of cells that carry mutations in *sub2* or the genes that encode the components of the THO complex conclude that the 3' end of genes is a site for assembly of novel protein-DNA complexes that include NPC components (Rougemaille et al., 2008). This suggests that THO and Sub2 act at a step after 3'-end processing.

Recruitment of mRNA export factors

The trafficking of most cargoes that move between the nucleus and the cytoplasm involves karyopherin-mediated receptors, and transport directionality is determined by a gradient of the GTP-bound state of the small GTPase Ran. Karyopherins that transport cargo from the nucleus to the cytoplasm, also referred to as exportins, bind both their cargo protein, and RanGTP in the nucleus. Once cargo and RanGTP are bound, the karyopherin interacts directly with the nuclear pore complex (NPC), resulting in nuclear export. After export into the cytoplasm is complete, RanGTP is hydrolyzed into RanGDP, stimulating release of the cargo, and successful transport into the cytoplasm. Karyopherins that transport cargo into the nucleus from the cytoplasm, also called importins, work in an opposing manner, binding only their cargo protein in the cytoplasm. Importins also mediate transport by direct interaction with NPC, and upon transport into the nucleus, the importin binds RanGTP, causing release of the cargo and completion of the import process. It is by this mechanism that most cellular cargo is transported in and out of the nucleus, including proteins, tRNA, microRNA and, the 40S and 60S ribosomal subunits.

The majority of mRNA export (later referred to as bulk mRNA export) is atypical in that it occurs by a mechanism that utilizes neither karyopherin receptors, nor the RanGTP system (Figure 1). Bulk mRNA is exported via the non-karyopherin heterodimer of Mex67 (*S. cerevisiae*; in human cells Nxf1 (also called TAP)) and Mtr2 (*S. cerevisiae*; in human cells Nxt1 (also called p15)). Mex67 was first discovered in *S. cerevisiae* in 1997 (Segref et al., 1997), and it's human homologue, Nxf1, in 1998 (Gruter et al., 1998). Early characterization showed that both the sequence of

Mex67/Nxf1, and its role in mRNA export were evolutionary conserved in *S. cerevisiae*, *D. melanogaster*, *C. elegans*, and *H. sapiens* (Herold et al., 2001; Herold et al., 2000; Katahira et al., 1999; Tan et al., 2000). Early work in the field also showed that while Mex67 is able to bind both poly(A^+) RNA, and the NPC directly, theoretically giving it the ability to act alone as the transport receptor. However, it can only function properly when bound as a heterodimer with the small protein, Mtr2 (Nxt1 in humans) (Santos-Rosa et al., 1998), and that this interaction is also evolutionarily conserved (Braun et al., 2001; Suyama et al., 2000; Wiegand et al., 2002). Interestingly, Mex67-Mtr2 is not known to be recruited directly to the mRNP, but rather utilizes adaptor proteins for its recruitment. The first of these adaptor proteins to be identified was the TREX component, Yra1. Early work in *S. cerevisiae* showed that Mex67-Mtr2 and Sub2 bind to the same domain in Yra1. As such, Sub2, which is recruited in the early phases of transcription, could recruit Yra1 to the mRNP complex and then be displaced by Mex67-Mtr2 (Strasser and Hurt, 2000). Furthermore, recent work in vertebrate cells shows that the binding of the Yra1 homologue, ALY, to mRNA is stimulated by the presence of the ATP-bound form of the Sub2 homologue, UAP56. This binding increases the ATPase activity of UAP56 (Taniguchi and Ohno, 2008). Moreover, Nxf1 binds mRNA-associated ALY, forming a ternary complex, and the RNA-binding affinity of Nxf1 is increased in the presence of ALY. Cells that express an altered form of Nxf1 that binds to ALY but not to mRNA have defective mRNA nuclear export (Hautbergue et al., 2008). Taken together, these data suggest a model whereby ATP-bound UAP56 (*S. cerevisiae* Sub2) recruits ALY (*S. cerevisiae* Yra1) to the mRNP complex. ATP hydrolysis by UAP56 (*S. cerevisiae* Sub2) triggers the transfer of the mRNA to ALY (*S.*

cerevisiae Yra1). Next, Nxf1-Nxt1 (*S. cerevisiae* Mex67-Mtr2) binds to ALY (*S. cerevisiae* Yra1), which causes another transfer event and results in an mRNP complex with bound export receptor, Nxf1-Nxt1.

Interestingly, while data from multiple model systems supports the canonical model of Nxf1-Nxt1/Mex67-Mtr2 recruitment above, other studies indicate that this is not the only mechanism by which Nxf1-Nxt1/Mex67-Mtr2 can get recruited to mRNA. Studies in *S. cerevisiae* have shown that in another multi-step process, the RNA-binding protein Npl3 can recruit Mex67 to poly(A^+) RNA (Gilbert and Guthrie, 2004). Npl3 is essential for both viability, and mRNA export, and is present in *S. cerevisiae* in both a phosphorylated, and non-phosphorylated state. Cells lacking the Npl3 kinase, Sky1, show a defect in nuclear poly(A^+) RNA export, indicating that phosphorylation is important for export, although phospho-Npl3 does not associate with poly(A^+) RNA in wild type cells in *in vivo* crosslinking experiments. However, phopho-Npl3 is found associated with poly(A^+) RNA in cells that harbor a mutant in the Npl3 phosphatase, *glc7-5*, showing that phospho-Npl3 can indeed bind poly(A^+) RNA. This suggests that phospho-Npl3 first binds pre-mRNA, and is susequently dephosphoryated prior to or coincident with polyadenylation. Strikingly, in *glc7-5* cells (the Npl3 phosphatase mutant), Mex67 recruitment to poly(A^+) RNA is reduced, and this effect is reproduced in cells harboring only a nonphosphorylatable form of Npl3. This data suggests a second model, whereby phospho-Npl3 is recruited to pre-mRNA, dephosphorylated before or coincident with polyadenylation, and recruits Mex67 after dephosphorylation.

Importantly, this demonstrates that there are at least two distinct mechanisms for Mex67 recruitment in *S. cerevisiae*, and that the post translational modification state of

other mRNA binding proteins can play a role in the recruitment process. Adaptor proteins other than ALY (Yra1 in *S. cerevisiae*) have also been shown to be able to recruit Nxf1 (Mex67 in *S. cerevisiae*) in higher eukaryotes. In tissue culture cells, siRNA knockdown of either Nxf1 (Herold et al., 2001; Herold et al., 2003; Wilkie et al., 2001) or UAP56 (Sub2 in *S. cerevisiae*) (Gatfield and Izaurralde, 2002; MacMorris et al., 2003) results in a dramatic block in mRNA export, whereas knockdown of ALY (Gatfield and Izaurralde, 2002; Katahira et al., 2009; Longman et al., 2003) results in a more modest defect, suggesting another adaptor(s) with redundant function. Recently, such a protein was discovered and called UIF, for UAP56 interacting factor (Hautbergue et al., 2008). Similar to ALY, UIF interacts with both UAP56, recruits Nxf1 to mRNA, and knockdown of UIF results in a block in nuclear mRNA export, indicating it plays a similar role to ALY in mRNA export. Taken together, these examples show that there are multiple mechanisms, utilizing multiple adaptor proteins, to recruit the Nxf1-Nxt1/Mex67-Mtr2 export receptor.

Interestingly, another layer of complexity exists regarding export receptor recruitment in higher eukaryotes. Whereas *S. cerevisiae* harbors only one isoform Mex67, higher eukaryotes have multiple isoforms of the Nxf proteins (*C. elegans* has two, *Drosophila* has four, and humans have six (Herold et al., 2000), although not all have been extensively characterized.) While all of the isoforms are highly conserved at the sequence level, there are striking differences in their functions. For example, Nxf2, but not Nxf1, interacts specifically with the fragile X mental retardation protein (FMRP) (Zhang et al., 2007). Strikingly, Nxf2 and FMRP interact specifically with Nxf1 mRNA and cause it to become destabilized in cell culture models. However, these reduced levels

of Nxf1 mRNA are only seen if both Nxf2 and FMRP are present, demonstrating that the effect is dependent on the two proteins working together. Consistent with these results, Nxf1 mRNA levels are lower in mouse hippocampal neurons and male germ cells, the tissues in which both Nxf2 and FMRP expression is the most robust. This lowered level of Nxf1, could be altering the expression pattern of these cells by altering the export of specific mRNAs. Since both FMRP and Nxf2 are needed to see this effect, and since expression of both is robust in neurons, this implicates the dual FMRP/Nxf2 destabilization of Nxf1 mRNA as a possible cause of the phenotype in fragile X mental retardation. Interestingly, mutations in another Nxf protein, Nxf5, have been reported in cases of human mental retardation (Frints et al., 2003; Jun et al., 2001). It is intriguing to think that these diseases are caused by altered mRNA export patterns, but whether or not this is the case awaits further investigation.

Notably, whereas bulk mRNA export occurs via the Mex67 (human Nxf1) pathway, a subset of transcripts is exported via the karyopherin CRM1 (Xpo1 in *S. cerevisiae)* (Figure 2). CRM1 mediates the export of unspliced, or partially spliced, HIV mRNA via a virally encoded adaptor protein, Rev (Neville et al., 1997). However, CRM1 is also involved in the export of a subset of endogenously encoded transcripts. CRM1 is not an RNA-binding protein, and thus must use an adaptor for export of endogenous mRNAs as it does for HIV transcripts. Some possible adaptors have been reported, including HuR for the export of *Cd83* and *Fos* mRNAs, and eIF4e for *cyclin D1* mRNA in human cells (Brennan et al., 2000; Culjkovic et al., 2006; Prechtel et al., 2006); however, adaptors for other potential CRM1-exported transcripts have yet to be discovered.

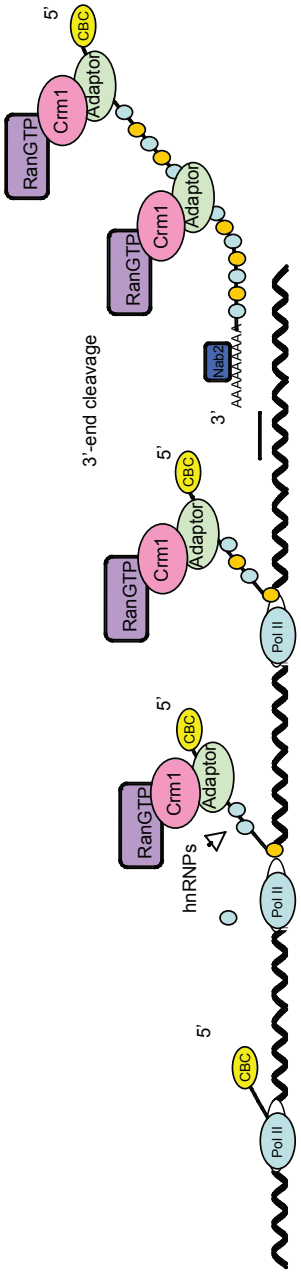

Figure 2: mRNA export mediated by Crm1. Transcripts exported via Crm1 are processed similarly to those exported by Mex67. This includes 5'-capping, and 3'-end formation. Crm1 interacts with the mRNA via an adaptor protein, although all known adaptors are not believed to be known. Crm1-mediated export is accomplished in a RanGTP dependent manner, with Crm1 binding both its adaptor protein and RanGTP in the nucleus.

While much progress has been made in this field in recent years, there are currently many outstanding questions yet to be addressed. It is not clear how many export receptors are needed for the transcript to be efficiently exported. Is there a defined threshold number, or do certain transcripts need more receptors than others? If some need more than others, what determines how many each need? Is it simply that longer transcripts need more receptors, or is there something inherent in each specific mRNA which determines how many will be recruited to it? It is also not clear if some species of mRNA use strictly one, or multiple mechanisms to recruit their export recetpor(s). ALY and UIF can be found on the same mRNA molecule, and both are necessary for efficient export, indicating that more than one receptor is needed for these mRNAs, and that they use two separate adaptors to recruit them. However it is unclear if this is true for each and every mRNA. In *S. cerevisiae*, for example, there is no UIF homologue. Do certain transcripts use only the Sub2/Yra1 mechanism, whereas others use Npl3? Or do some transcripts use both?

mRNA surveillance and nuclear retention of incompletely processed transcripts

In order to ensure that its genes are faithfully expressed, cells have evolved mechanisms to eliminate damaged, or improperly processed mRNAs. The first one of these mechanisms that an mRNA must avoid is nuclear retention and degradation by the nuclear exosome. In order to become export competent, mRNAs must successfully undergo complete and proper nuclear processing, including 5' capping, 3' end formation, polyadenylation, and splicing (if the transcript contains introns). If processing is not complete, transcripts can be recognized by the nuclear surveillance machinery, and

retained in the nucleus, thus blocking their expression. Once trapped in the nucleus, they can be recognized and degraded by the nuclear exosome (Figure 3). An early demonstration of this retention came from a study in *S. cerevisiae* with cells expressing mRNAs with both U1A hairpins engineered into their 3'-ends and U1A binding protein fused to green fluorescent protein (GFP) (Brodsky and Silver, 2002). In this system, the U1A-GFP binds the U1A hairpins, allowing for indirect fluorescent visualization of a single species of transcripts in living cells. Utilizing the ability to focus on a single mRNA species at a time, transcripts with distinct features were analyzed under multiple conditions. Interestingly, while all observed transcripts were retained in the nucleus in strains with mutations affecting genes encoding proteins in the mRNA export machinery, or certain (NPC) proteins, only intron containing transcripts were retained in cells defective for splicing. Further, all transcripts observed were retained in the nucleus in cells defective in 3' end formation, or polyadenylation. This study demonstrates that when transcripts are not properly processed, they are not exported.

Further work in the field has show that this retention is mediated by the nuclear exosome. The exosome is a complex of 3'-5' exonucleases, and functions in both the nucleus and cytoplasm to degrade mRNA targeted by the surveillance machinery. In *S. cerevisiae*, the nuclear exosome has one necessary protein component that the cytoplasmic exosome does not, called Rrp6. As such deletion of *RRP6* allows for specific inactivation of the nuclear exosome. Strikingly, multiple studies have shown that the accumulation of mRNA that is observed in mutants defective in transcription, 3' end processing and polyadenylation is due to retention at or near the site of transcription. Retention is no longer seen in cells lacking *RRP6* (Hilleren et al., 2001; Libri et al., 2002;

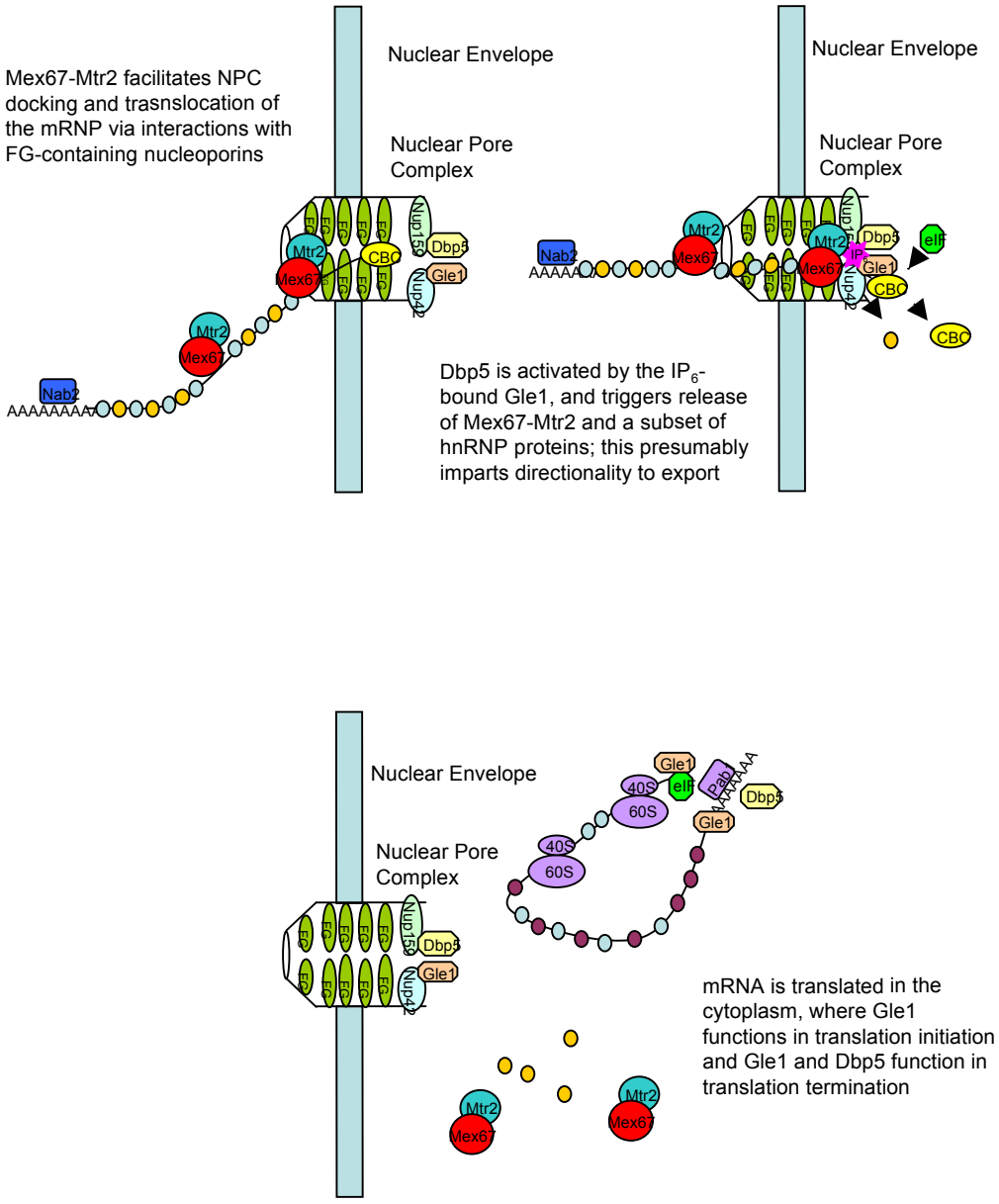

Figure 3: Nuclear export of mRNA occurs through the nuclear pore complex. mRNA is docked to the nuclear pore complex via a its export receptor. Transcripts are passed through the pore complex, and released in the cytoplasm where they can be translated. Specific steps are detailed in the figure.

Zenklusen et al., 2002). Taken together, this suggests a mechanism whereby the cell senses defective mRNAs, retains them at their site of transcription, and degrades them in a 3'-5' manner, thus blocking protein production of what could be faulty messages.

Interestingly, while mRNAs that have defects in their transcription, or 3' end processing are retained at their site of transcription, and detected by the exosome, unspliced mRNA is retained by a completely distinct mechanism. Unlike mRNA with defects in their 3' ends, unspliced transcripts are retained at the nuclear periphery, not at the site of transcription. Also, retention is not mediated by the nuclear exosome, but by a nuclear envelope associated protein, called Mlp1 (Galy et al., 2004). Mlp1 is a conserved protein (known as TPR in higher eukaryotes) that associates with the nuclear envelope, and nuclear face of the nuclear pore complex. In cells lacking *MLP1*, unspliced mRNA is exported into the cytoplasm. Conversely, in cells overexpressing *MLP1*, intron-containing transcripts are preferentially retained in Mlp1 containing foci. The precise molecular mechanism for Mlp1-mediated retention is not currently clear, although it has been hypothesized that Mlp1 retains unspliced mRNA that has formed the commitment complex, CC1, but not proceeded through the splicing process. In line with this, both CC1 formation and Mlp1-mediated retention are dependent on the 5' splice site. Also, wild type cells leak ~5% of unspliced mRNA into the cytoplasm, which is roughly the same percentage of cells that escape early intron recognition, and thus do not form CC1 (Rosbash and Seraphin, 1991).

These studies illustrate the importance of early mRNA processing steps on nuclear export, as well as the complexity of the export process. For export to be successfully executed, all prior processing steps must be completed correctly, and as

such, in order to completely understand the export process, one must have a complete understanding of the earlier processing events on which it is dependent.

Docking to and passage through the NPC

Following completion of proper nuclear processing and the recruitment of an export receptor, an mRNP complex is considered to be export competent. This export-competent mRNP complex is specifically targeted to the NPC via its export receptor (Figure 4). For some transcribed genes, the positioning of the respective chromatin region near the NPC might facilitate export by physically linking the processes (reviewed in (Akhtar and Gasser, 2007). Such a mechanism is described in an early gene-gating model (Blobel, 1985).

The export receptor docks at the NPC by interacting with a discrete class of NPC proteins known as the FG-Nups, which have been thus designated based on the presence of distinct domains containing multiple repeats of the amino acids phenylalanine (F) and glycine (G), separated by characteristic spacer sequences. Specific subtypes of FG repeats include FxFG and GLFG (L, leucine). Approximately one third of the 30 proteins that make up the NPC are FG-Nups. They assemble into peripheral substructures on the cytoplasmic and nuclear NPC faces and throughout the NPC central channel. Both the karyopherin receptors and Mex67-Mtr2 mRNA export receptors directly bind the FG repeats in domains that are distinct from their respective cargo or mRNP binding-domains (reviewed in (Stewart, 2007) Thus, the export receptor serves to bridge the interaction of the mRNP complex and the NPC.

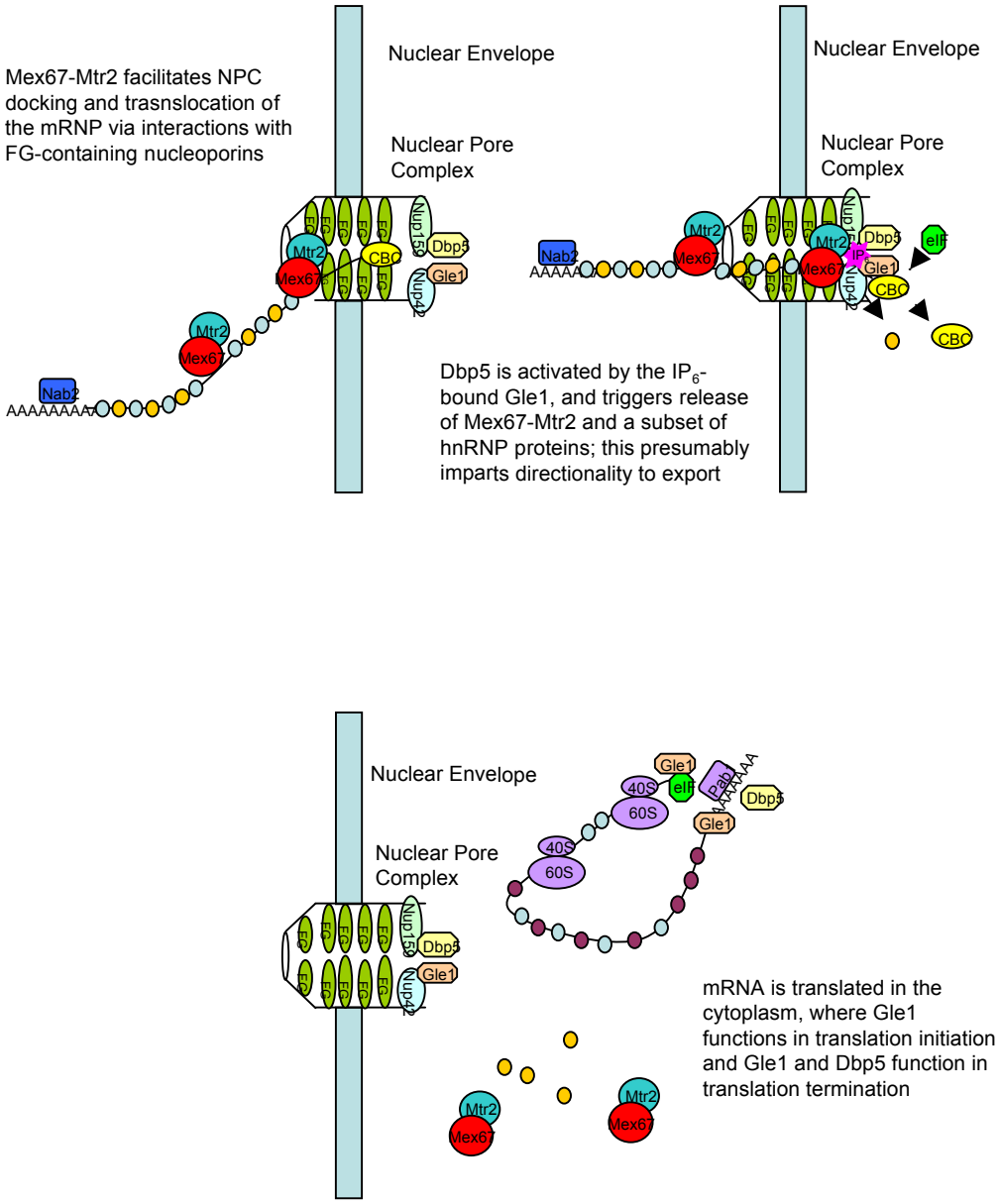

Figure 4: Nuclear export of mRNA occurs through the nuclear pore complex. mRNA is docked at the nuclear pore complex through its export receptor. Transcripts are passed through the pore complex into the cytoplasm where they can be translated. Specific steps are detailed in this figure.

Binding of the export receptor to the FG-Nups is required for NPC docking and translocation of the mRNP complex. Studies of the Balbiani ring mRNP complex, a mRNP complex the salivary glands of the insect *Chironomus tentans* that is large enough visualize by electron microscopy, show that the 5' end of the mRNP complex docks first to the NPC nuclear face, with extrusion through the NPC proceeding with the 5' end leading (Visa et al., 1996). Whether this '5' first' mechanism is utilized by smaller mRNPs complexes is unknown (the Balbiani ring mRNP measures ~50nm in diameter (Mehlin et al., 1992)). The binding of transport receptors to the FG-Nups is thought to mediate the movement of the mRNP complex through the NPC by some type of facilitated diffusion mechanism (Weis, 2007). It is commonly agreed that the FG-Nups themselves do not provide directionality to translocation. Interestingly, however, work has revealed that a specific subset of the FG-Nups is required for Mex67-Mtr2-mediated mRNA export in *S. cerevisiae* (Terry and Wente, 2007). Crucial FG-Nup binding sites for mRNA export are found on the nucleoplasmic face and in the central channel of the NPC. Moreover, Mex67-Mtr2 apparently utilizes a set of FG-Nups distinct from those used by several key karyopherins.

Release into the cytoplasm and links to translation

The final step of the NPC translocation of mRNP complexes involves directional release into the cytoplasm. Because mRNA export mediated by Mex67-Mtr2 is not dependent on the RanGTP gradient, an alternative mechanism must determine directionality. Recent work in *S. cerevisiae* has provided compelling evidence that the directionality of cytoplasmic release is determined by the function of two conserved,

essential mRNA export factors, Dbp5 and Gle1, and soluble inositol hexakisphosphate (IP_6) (Alcazar-Roman et al., 2006; Weirich et al., 2006). Dbp5 is an RNA-dependent ATPase of the DEAD-box protein family, and binds to the NPC cytoplasmic face by interacting with the NPC protein Nup159 (in *S. cerevisiae*; Nup214/CAN in human cells) (Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998; Weirich et al., 2004). Gle1 specifically binds IP_6 and docks to a neighboring NPC protein Nup42 (in *S. cerevisiae*; hCG1 in human cells) (Alcazar-Roman et al., 2006; Kendirgi et al., 2005; Murphy and Wente, 1996; Strahm et al., 1999). As an mRNP complex reaches the cytoplasmic side of the NPC, it associates with Gle1 and Dbp5. It is possible that Dbp5 is also co-transcriptionally recruited to the mRNP complex (Zhao et al., 2002). IP_6-bound Gle1 stimulates the ATPase activity of Dbp5, thereby converting Dbp5 from an ATP to ADP bound state (Tran et al., 2007). It is thought that a conformational change induced by the Dbp5-ATP to Dbp5-ADP switch triggers the removal of a subset of proteins from the mRNP complex, including the export receptor Mex67 and the poly(A^+)-binding protein Nab2 (von Moeller et al., 2009). This changes the composition of the proteins in the mRNP complex (Lund and Guthrie, 2005; Tran and Wente, 2006; Tran et al., 2007). As such, the spatially controlled remodeling of the mRNP being exported confers the directionality of export as the export receptor is removed. The number of Dbp5 ATP hydrolysis cycles that occur per mRNP complex transported, and the mechanism for selective remodeling, are unknown.

The proteins that are removed by Dbp5 are recycled by being imported into the nucleus for another round of mRNA export. In addition, as the mRNP complex enters the cytoplasm, specific cytoplasmic mRNA-binding proteins are incorporated. Studies of the

export of the Balbiani ring mRNP complex have found that the binding of cytoplasmic factors occurs immediately as the 5' end of the transcript enters the cytoplasm; the cap binding complex (CBC) is replaced with eIF4e, ribosomes bind and translation begins before the entire mRNP has been extruded from the NPC (Daneholt, 2001). Interestingly, Dbp5, Gle1 and IP_6 also have roles in translation (Bolger et al., 2008; Gross et al., 2007). Assembly of the termination complex on the mRNA may require Gle1-IP_6-dependent stimulation of Dbp5, and Gle1 also has a distinct role in translation initiation. These links to translation further show the inherent connections between steps in gene expression.

Differential requirements for export of individual mRNAs

While, with few exceptions, most mRNAs are subject to similar nuclear processing steps, multiple lines of evidence exist that indicate that different mRNAs are bound to different sets of mRNA-binding proteins, including proteins involved in nuclear export. An early study demonstrating this focused on the mRNA export receptor, Mex67, and its adaptor protein, Yra1, and asked whether each of these proteins was bound to the same, or distinct sets of mRNAs (Hieronymus and Silver, 2003). To address this, epitope-tagged versions of each protein were purified from *S. cerevisiae*, and co-purifying mRNA was analyzed by microarray. This showed that Mex67 and Yra1 interact with overlapping, but distinct pools of mRNAs, and that the differing pools of mRNA in some cases were comprised of transcripts encoding proteins of distinct biological functions. For example, Yra1, but not Mex67, was enriched on transcripts encoding proteins involved in carbohydrate metabolism, and conversely Mex67, but not

Yra1 was enriched on transcripts encoding integral membrane proteins. Some classes of mRNA were enriched for both Yra1 and Mex67, including transcripts encoding ribosomal proteins, and translation factors. This suggests that even though Mex67 and Yra1 are both essential for bulk mRNA export, with mutants in both having severe export defects detected by *in situ* hybridization with an oligo d(T) probe to detect poly(A^+) RNA, either may be dispensable with regards to export of certain specific transcripts. In support of the idea that mRNA export can be carried out independently of Yra1 or Mex67, each of these genes, despite being highly conserved, has been found to be non-essential for export in other species. For example, Mex67/Nxf1 is essential for bulk mRNA export in *S. cerevisiae*, *Drosophila*, and in human cell culture models (Braun et al., 2001; Farny et al., 2008; Segref et al., 1997; Wiegand et al., 2002), but is not essential for export is the fission yeast, *Schizosaccharomyces pombe* (Yoon et al., 2000). Yra1 is also essential for export in *S. cerevisiae* (Strasser and Hurt, 2000), but is not in *C. elegans* or *Drosophila* (Gatfield and Izaurralde, 2002; Longman et al., 2003).

Another study addressed this in a similar manner using three hnRNP proteins from *S. cerevisiae*, Npl3, Nab2, and Nab4 (Guisbert et al., 2005). This study found that, similar to Mex67, and Yra1, each of these three proteins bound to a different subset of mRNAs. Interestingly, pools of mRNAs encoding genes of specific function were again isolated with specific mRNA-binding proteins. Nab2 was found bound to transcripts encoding proteins involved in transcription; Npl3 was found bound to transcripts encoding ribosomal subunits; and Nab4 was found bound to transcripts encoding proteins involved in multiple different processes including alcohol metabolism, carboxylic acid metabolism, ergosterol metabolism, and branched chain amino acid metabolism.

Demonstrating that these unique hnRNP:mRNA interactions have biological relevance, *nab4* mutants have decreased sensitivity to sulfometuron methyl (SMM), a drug that causes depletion of branched amino acids, but not to other stress condition, including high salt conditions, and hypo-osmolarity. As with Mex67 and Yra1, this suggests that while Nab2 and Npl3 are both essential factors, perturbation of which results in severe mRNA export defects, they may only be essential for export of certain subsets of transcripts, while being dispensable for others.

Consistent with this, mutations in *TOM1*, an E3 ubiquitin ligase in *S. cerevisiae*, results in a lack of nuclear export of Nab2, but not Npl3 (Duncan et al., 2000). Both Nab2 and Npl3 are only known to be exported from the nucleus bound to mRNA, while active RNA polymerase II transcription is occuring. As such, cells with mutations in *TOM1* present a case where Nab2-bound transcripts, but not Npl3-bound transcripts, are specifically retained in the nucleus, indicating that are at least two specific pools of mRNA with specific export requirements. A third study has taken a more global approach, and performed similar analysis on forty different RNA-binding proteins. Similar results were found, showing that different mRNA-binding proteins bind different transcripts (Hogan et al., 2008). Interestingly, by taking a more global approach, this work has showed that most mRNAs are bound to multiple binding proteins, although the set of proteins on each specific mRNA can vary. While not surprising, this does raise some intriguing questions, such as, how many mRNA-binding proteins are essential for the export of each mRNA? And, how similar is this cohort of proteins for each individual mRNA?

Further evidence of differential requirements for export comes from a genetic screen in *Drosophila* (Farny et al., 2008). Farny and coworkers performed a genome wide siRNA screen to identify genes that affected mRNA export, as seen by accumulation of poly(A^+) RNA after knockdown. From this, they identified 72 individual genes, and compared them to the 45 genes in *S. cerevisiae* that are known to have mRNA export defects. Interestingly, only 18 of these genes showed mRNA export defects in both species, leaving 27 genes responsible for defects in *S. cerevisiae* that were not show to have defects in *Drosophila*, and 54 genes responsible for defects in *Drosophila*, that aren't in *S. cerevisiae*.

Taken together, these studies illustrate the complexity of mRNA export. While the factors discussed above play clear roles in the process of mRNA export, and are highly conserved in many cases, how they all work in concert to accomplish the task at hand remains unclear. Classically, mutants in genes encoding proteins with a suspected role in mRNA export have been verified by showing accumulation of poly(A^+) RNA in the nucleus. While this methodology can determine if there is a block in overall export, it cannot determine if there is a block in the export of all individual mRNA species. The studies above provide strong evidence that while we have likely identified most of the factors involved in the overall process of mRNA export, elucidation of the precise function and necessity of each of these on a transcript-to-transcript, species-to-species, and potentially a cell type-to-cell type basis will require further investigation.

Cellular response, and mRNA export during heat shock

When cells are exposed to extreme environmental stress conditions, they often undergo multiple rapid responses in order to best cope with the stress at hand. One such example is exposure to elevated temperatures, known as heat shock, and the subsequent cellular heat shock response. While the heat shock response is evolutionarily conserved from bacteria to humans, further discussion will focus more specifically on the response in *S. cerevisiae*. When exposed to high temperatures, *S. cerevisiae* cells undergo a rapid change in their gene expression profiles, whereby their normally expressed genes are downregulated, and production of a set of proteins known is heat shock proteins (HSPs) is dramatically upregulated. Heat shock proteins are essential for cellular survival during heat shock, acting as molecular chaperones to help stabilize proteins which can lose their folding conformations due to the increased thermal energy (Kregel, 2002). In order to accomplish this switch in expression patterns, heat shock genes are rapidly and robustly transcribed, exported from the nucleus and translated. Conversely, non-heat shock transcripts are retained in the nucleus, and not efficiently exported into the cytoplasm to be translated. By this mechanism, cells are able to utilize the translation machinery specifically to produce the proteins they need to survive the thermal stress, while not wasting energy expressing proteins that are not immediately needed for survival. However, in order to accomplish this change in export patterns, cells must have a way to efficiently and selectively export heat shock mRNAs, while retaining non-heat shock mRNAs.

Interestingly, all of the previously discussed studies regarding differential protein requirements for different mRNA species were carried out under non-stressed growth conditions, indicating that those separate pathways for export occur simultaneously during normal growth conditions. However, when cells undergo a heat shock response, they specifically target certain mRNAs for export, and others for retention, utilizing a specific mRNA export pathway(s), while inhibiting the others. Strikingly, while a core set of mRNA export factors in *S. cerevisiae* have roles in both heat shock, and not-heat shock mRNA export, there are others that have roles in one but not the other. Among the proteins involved in both heat shock and non-heat shock export are the mRNA export receptor, Mex67 (Hurt et al., 2000; Segref et al., 1997), and the NPC-associated export factors Gle1, and Dbp5 (Murphy and Wente, 1996; Snay-Hodge et al., 1998; Tseng et al., 1998). Interestingly, while Mex67 is necessary for both non-heat shock and heat shock mRNA export, both of its known adaptor proteins, Yra1 and Npl3, are both dispensable for the latter (Krebber et al., 1999; Rollenhagen et al., 2007). This indicates that there is another, yet to be discovered, mechanism for Mex67 recruitment, which occurs for heat shock mRNA. Conversely, while Yra1 and Npl3 are necessary for only non-heat shock mRNA export, the nuclear pore protein Nup42 is essential for only heat shock mRNA export. Nup42 is an FG-nucleoporin, which resides on the cytoplasmic face of the NPC, and has a binding site for the export factor, Gle1 (Strahm et al., 1999; Stutz et al., 1996). *NUP42* is a non-essential gene, and deletion does not result in a bulk poly(A^+) mRNA export defect, but does result in a defect. However, a strain lacking *NUP42* has a defect in the export of heat shock mRNA (Saavedra et al., 1997). The mechanism underlying this discrepancy is currently not known. Taken together, this suggests a model whereby

both heat shock and non-heat shock mRNA similarly utilize the mRNA export receptor Mex67, and require the remodeling activity of Gle1/Dbp5 at the NPC for cytoplasmic release However heat shock mRNA specifically utilizes a distinct mechanism to recruit Mex67, and does not require a set of a mRNA binding proteins necessary for bulk poly(A^+) RNA export.

Another cellular response during heat shock in *S. cerevisiae* is the induction of the cell wall integrity mitogen-activated protein (MAP) kinase cascade. MAP kinase cascades are evolutionarily conserved signaling pathways, that can be activated by a number of extracellular stimuli including, cytokines, osmotic stress, and heat shock (Chen and Thorner, 2007; Pearson et al., 2001). Each MAP kinase cascade consists of three kinases, a MAPKKK or MEKK, a MAPKK or MEK, and a MAPK. Upon activation of a given cascade, the MAPKKK phosphorylates the MAPKK on two serine or threonine residues (Alessi et al., 1994; Zheng and Guan, 1994). Once phosphorylated, the MAPKK becomes active, and phosphorylates the MAPK on a tyrosine and a serine or threonine residue, activating the MAPK. The activated MAP kinase can then phosphorylate its target proteins, which can include other kinases, and transcription factors. Phosphorylation of these target proteins in turn elicits the cascade's biological response.

In *S. cerevisiae* there are five distinct MAP kinase cascades, each of which is activated by different external stimuli (Gustin et al., 1998). One of these, the cell wall integrity (CWI) MAP kinase pathway, is activated to mediate cell wall synthesis, or to respond to respond to stress to the cell wall (Figure 5). Signals that can initiate this pathway include mating pheromone and cell cycle cues, which signal for the need to

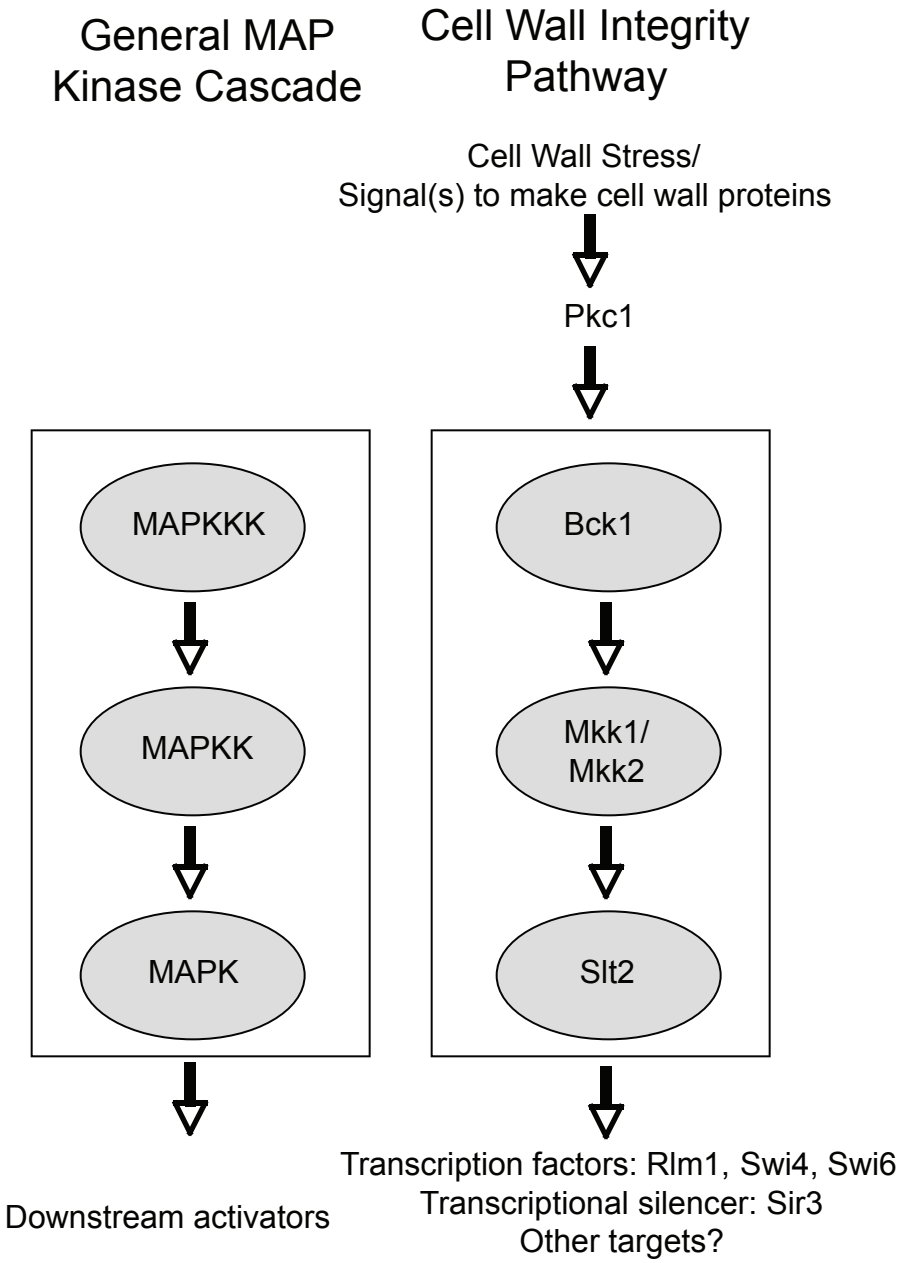

Figure 5: The cell wall integrity MAP kinase pathway. The *Saccharomyces cerevisiae* cell wall intergrity (CWI) MAP kinase cascade is activated in response to cell wall stress, and signals to make new cell wall proteins. The pathway is activated by Pkc1, and consists of the MAPKKK, Bck1, two redundant MAPKKs, Mkk1, and Mkk1, and the MAPK, Slt2. Activation of the pathway results in phosphorylation of the transcription factors, Rlm1, Swi4, and Swi6, and the transcriptional repressor, Sir3.

make more cell wall proteins, and heat shock or exposure to lytic enzymes, which signal for the need to repair a damaged cell wall. The CWI MAP kinase pathway consists of Bck1 (MAPKKK), Mkk1 and Mkk2 (two functionally redundant MAPKKs), and Slt2/Mpk1 (the MAPK, later referred to as Slt2). While this pathway is necessary for multiple biological responses, the focus here will be specifically on the pathway's function during heat shock. During heat shock, the yeast cell wall becomes stressed due to the increase in thermal energy. This stress in the cell wall can be sensed by five plasma membrane proteins, Wsc1, Wsc2, Wsc3, Mid2, and Mtl1, which in turn leads to activation of the yeast protein kinase C homologue, Pkc1. Precisely how this stress in the cell wall is sensed by plasma membrane proteins is unclear, although it is thought to be due stretching of the plasma membrane, and perturbation of the connection of the cell wall to the plasma membrane. This then leads to Pkc1 activation, which then phosphorylates Bck1, initiating the CWI MAP kinase cascade.

Upon pathway activation, the MAPK Slt2 has been shown to phosphorylate the transcription factors, Rlm1 and the SBF transcription factor subunits, Swi4, and Swi6, leading to an increase in transcription of cell wall genes (Chen and Thorner, 2007). Upregulation of cell wall genes is essential for survival during heat shock. Cells lacking *SLT2*, and thus not expressing increased amounts of cell wall proteins, lyse in response to heat shock. Interestingly, while the paradigm for mRNA export during heat shock is that heat shock mRNAs are exported and non-heat shock mRNAs are retained in the nucleus, non-heat shock transcripts induced by Slt2 seemingly avoid this retention, as their expression is necessary for survival during shock. Of note, Slt2 has also been shown to

phosphorylate Sir3, a protein involved in transcriptional silencing, indicating that it acts as more than just an activator of transcription (Ray et al., 2003).

Links to disease and development

Based on its roles in proper gene expression, an increasing number of links for nuclear mRNA export to human disease and development are emerging. Distinct pathophysiological states are correlated with defective mRNA export mechanisms, including 1) mutation of genes encoding export factors or mRNA binding proteins, 2) mutations in genes that inhibit the proper export of their own transcripts, and 3) down regulation or hijacking of the endogenous mRNA export machinery by viruses to allow specific viral gene expression. Examples of each are discussed below.

Recent work shows that LCCS1, a fetal motor neuron disease, is linked to mutations in human *GLE1* (Nousiainen et al., 2008). The molecular mechanism for the Gle1 defect and the basis for the neuron-specific defects await analysis. In addition, the fragile X mental retardation protein, FMRP, interacts with an Nxf1 homologue, Nxf2, and destabilizes *Nxf1* mRNA in neurons, presumably leading to a decrease in the protein levels of this mRNA export factor (Zhang et al., 2007). Such downregulation of the mRNA export receptor Nxf1 could potentially alter the pool of exported transcripts if Nxf1 and Nxf2 target different subsets of the mRNAs, or if the two proteins export some transcripts more efficiently than others.

Mutations of genes encoding specific export factors have also been shown to play a role in vertebrate development. Nxf2 is required for cardiac development in zebrafish (Huang et al., 2005), although the precise mechanism for this cardiac specific phenotype

is not fully known. Another disease associated with aberrant mRNA export is osteogenesis imperfecta type I (OI) (Hurt and Silver, 2008). In OI patients, a mutation in one of the two genes encoding collagen, *COL1A1* or *COL1A2*, results in bone fragility. Whereas multiple mutations in collagen genes have been found to cause OI, a specific splice site mutation in one cohort of patients results in defective splicing and nuclear retention of collagen mRNA; thus, lowering collagen expression (Johnson et al., 2000; Stover et al., 1993).

Myotonic dystrophy (DM) type I is also caused by a mutation that results in nuclear accumulation of its mRNA transcripts. DM is a severe muscle wasting disorder, which results in skeletal muscle myotonia, and degeneration, and can eventually lead to death. The most common form of DM in adults (DM type I) is caused by a mutation which results in an abnormal CUG expansion in the 3' UTR in the DM protein kinase (DMPK) mRNA which results in it being retained in the nucleus. Interestingly, DM is an autosomal dominant disease, with the disease state not caused not by reduced expression of DMPK, but in *trans* by sequestration of splicing factors on the retained transcript, including the muscle-blind protein family 1 (MBNL1), and CUG-binding protein 1 (CUGBP1) (Charlet et al., 2002; Mankodi et al., 2002; Phillips et al., 1998; Savkur et al., 2001). This sequestration of splicing factors prevents them from carrying out their normal functions, which in this case results in lowered levels of splicing of specific muscle-related transcripts, and the DM disease state (Dansithong et al., 2005).

Nucleocytoplasmic transport and mRNA nuclear export are essential for the proliferation of viruses that depend on nuclear replication (Fontoura et al., 2005; Greber and Fornerod, 2005). This includes DNA tumor viruses, RNA viruses, and RNA

retrotransposons, retroviruses and some negative-sense viruses. In regard to viral mRNA export, there are several distinct mechanisms that target the endogenous mRNA export machinery (Cullen, 2003; Fontoura et al., 2005). For example, human immunodeficiency virus (HIV)-encoded mRNA is exported via the karyopherin Crm1 (Cullen, 2003; Greber and Fornerod, 2005), but also potentially utilizes the endogenous DEAD-box protein DDX3 for efficient mRNA export (Yedavalli et al., 2004). In contrast, to efficiently export its mRNA, influenza virus expresses the NS1 protein that forms inhibitory complexes with essential export factors including Nxf1-Nxt1, and blocks export of endogenous mRNA (Satterly et al., 2007). In this manner, the influenza virus ensures that its transcripts are preferentially exported. More defined and continued analyses of how viruses utilize and target the endogenous mRNA export machinery could potentially yield targets for future drug development.

Concluding remarks

Many outstanding questions remain in the field of mRNA export. It is currently unclear whether the transport of every mRNA occurs via the paradigms outlined above. The precise biochemical determinants of an export-competent mRNP complex, and whether they are the same for every mRNA, have not been not fully defined. Genetic and biochemical studies in *S. cerevisiae* have hinted that there are distinct, differential requirements for export competency that are defined by the presence of different mRNA-associated hnRNPs (Duncan et al., 2000; Guisbert et al., 2005).

Another outstanding question in the field is whether mRNA export depends on the same core set of export factors and occurs in precisely the same manner in every

organism, or in every cell type. Although the export factors discussed here are highly conserved, studies of different model organisms have provided potentially conflicting reports of their absolute necessity. For example, Mex67 is essential for mRNA export in the budding yeast *S. cerevisiae* (Segref et al., 1997), but not in the fission yeast, *Schizosaccharomyces pombe* (Yoon et al., 2000); Yra1 is essential for export in *S. cerevisiae* (Strasser and Hurt, 2000), but not in *Drosophila* or *Caenorhabditis elegans* (Gatfield and Izaurralde, 2002; Longman et al., 2003). Further, specific cell types have been demonstrated to be more sensitive to mutation of specific mRNA export factors, as is the case with LCCS1 and fragile X mental retardation, where neurons are particularly sensitive to mutations in Gle1 and Nxf2, respectively. It also currently not clear how mRNA export is affected as a response to different cellular stress conditions. While there is a specific response to export of heat shock mRNA during growth at elevated temperatures in *S. cerevisiae*, the same response is not elicited when cells are subjected to high osmolarity stress. Therefore, a thorough understanding of mRNA export will require analysis of species-specific differences, cell-type specific differences, as well as stress response specific differences. Future studies offer the promise of insights into the mRNA export mechanism that will impact studies of cell biology, virology, and gene expression regulation in both normal human development and pathophysiology.

CHAPTER II

THE MAP KINASE SLT2 REGULATES NUCLEAR RETENTION OF NON-HEAT SHOCK mRNAs DURING HEAT SHOCK-INDUCED STRESS

Introduction

In eukaryotes, intracellular signaling events are essential for transmitting information from the external environment to gene expression networks (reviewed in Chen and Thorner, 2007). This communication is particularly important for allowing rapid cellular adaption and survival during stress conditions. It is well established that environmental stress results in an evolutionarily-conserved global alteration of the nuclear mRNA export pathway (Bond, 2006; Gallouzi et al., 2000; Izawa et al., 2005; Krebber et al., 1999). In response to heat shock, transcripts from genes encoding heat shock proteins (hsp) are exported whereas most non-hsp poly(A^+) mRNAs are retained and accumulate in the nucleus (Saavedra et al., 1996; Saavedra et al., 1997). Together with the coincident increased transcription of heat shock genes, the retention of non-hsp mRNAs results in a coordinated mechanism for the rapid production of the heat shock proteins essential for survival and stress recovery (Bond, 2006). How signal transduction pathways modulate the mRNA export mechanism is not fully defined.

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For mRNA export under normal growth conditions, the transcription and mRNA export complex (TREX) is critical for coupling mRNA biogenesis with messenger ribonucleoprotein particle (mRNP) packaging to allow formation of an export competent mRNP (Abruzzi et al., 2004; Strasser and Hurt, 2001; Strasser et al., 2002). The TREX complex includes the essential mRNA binding protein Yra1 and the export receptor Mex67-Mtr2 in *S. cerevisiae* (TAP/NXF1-p15/NXT1 in vertebrates) (Herold et al., 2001; Segref et al., 1997; Strasser et al., 2000). Recruitment of Mex67-Mtr2 appears to be the penultimate step, which stimulates release of the mature mRNP from chromatin-associated biogenesis factors and the transition to early mRNA export steps (Johnson et al., 2009; Qu et al., 2009). Association of Mex67-Mtr2 with the mRNP is key for export, and directly mediates subsequent targeting to nuclear pore complexes (NPCs) in the nuclear envelope and NPC translocation via interactions between Mex67-Mtr2 and NPC proteins (nucleoporins, Nups) (reviewed in Kohler and Hurt, 2007) (Terry et al., 2007). Thus, defining the mechanisms that control interactions between mRNA binding proteins and Mex67-Mtr2 will likely reveal important regulatory steps.

Several essential mRNA binding proteins have been implicated in the mRNA export mechanism (Kelly and Corbett, 2009). In the budding yeast *S. cerevisiae*, this includes Npl3, Yra1, and Nab2 (Hector et al., 2002; Lee et al., 1996; Strasser and Hurt, 2000). These factors couple mRNA biogenesis steps such as transcriptional elongation, pre-mRNA splicing and 3' end formation with assembly of an export-competent mRNP (Apponi et al., 2007; Deka et al., 2008; Gallardo et al., 2003; Kress et al., 2008; Zenklusen et al., 2002). Other key *S. cerevisiae* components involved in early mRNA export are Mlp1 and Mlp2 (Green et al., 2003; Vinciguerra et al., 2005). The Mlp

proteins associate with the NPC and promote docking of mRNPs to the nuclear envelope. Studies have shown that the poly(A^+) binding protein Nab2 interacts directly with Mlp1, with loss of the Nab2-Mlp1 interaction enhancing the growth and mRNA export defects of *mex67* mutants (Fasken et al., 2008; Green et al., 2003). This suggests that a Nab2-Mlp1 step is central to efficient mRNA export. Importantly, the Mlp proteins also function in a nuclear quality control mechanism that acts to retain unspliced or aberrantly processed mRNAs in the nucleus (Galy et al., 2004; Palancade et al., 2005; Vinciguerra et al., 2005). Although the precise role for the Mlp proteins in quality control is unknown, genetic and biochemical evidence suggests that Nab2 and Yra1 are linked to this process.

Many of the factors required for normal, non-hsp mRNA export are also essential for hsp export; specifically, Mex67, the DEAD-box protein Dbp5, and the Dbp5-activator Gle1 (Alcazar-Roman et al., 2006; Hurt et al., 2000; Murphy and Wente, 1996; Rollenhagen et al., 2004; Saavedra et al., 1997; Segref et al., 1997; Snay-Hodge et al., 1998; Tseng et al., 1998; Vainberg et al., 2000; Weirich et al., 2004). Altering the function of these proteins results in the impaired export of all mRNAs, consistent with their general roles in the mRNA export process. In contrast, hsp mRNA export is independent of some factors required for normal mRNA export including the mRNA binding proteins Yra1 and Npl3 (Krebber et al., 1999; Rollenhagen et al., 2007). Furthermore, hsp mRNAs show a requirement for the NPC protein Nup42 whereas this factor is dispensable for efficient mRNA export under normal growth conditions (Rollenhagen et al., 2004; Saavedra et al., 1996; Saavedra et al., 1997; Stutz et al., 1997; Vainberg et al., 2000). Given that several studies have found that specific mRNA-binding

proteins associated with transcripts whose protein products are functionally linked, regulating the mRNP composition for transcripts might be a mechanism for controlling mRNA export (Farny et al., 2008; Guisbert et al., 2005; Hieronymus and Silver, 2003; Hogan et al., 2008). However, it is not clear how mRNP composition is affected during heat shock or if other molecular signals differentiate between hsp and non-hsp mRNAs during mRNA export.

The stress induced by heat shock is known to trigger a cascade of intracellular signaling events, including stimulation of the mitogen-activated protein kinase (MAPK) pathways (Chen and Thorner, 2007). *S. cerevisiae* has five distinct MAP kinase pathways, each of which can be activated by specific extracellular stimuli. The Bck1-Slt2/Mpk1 pathway is specifically initiated by certain forms of cellular stress, including cell wall stress and heat shock (Martin et al., 1993; Torres et al., 1991; Truman et al., 2007). When cells undergo heat shock, Pkc1 initiates the kinase cascade by phosphorylating the MAPKKK (Bck1) which then phosphorylates two redundant MAPKKs (Mkk1 and Mkk2) for phosphorylation of the MAPK (Slt2/Mpk1) (Levin et al., 1994). Activated Slt2 phosphorylates both transcriptional activators and repressors, altering the gene expression pattern to allow cell survival (Dodou and Treisman, 1997; Igual et al., 1996; Ray et al., 2003). Interestingly, the vertebrate ortholog of the Mlp proteins, designated Tpr1, is a target of a MAP kinase pathway in vertebrate cells (Skaggs et al., 2007; Vomastek et al., 2008). Thus, there is a potential connection between these signaling pathways and mRNA export during heat shock.

In this study, we demonstrate that the essential poly(A^+) mRNA-binding protein Nab2 is a target for heat shock-dependent phosphorylation by the MAP kinase Slt2.

Unlike wild type cells, *slt2* null (*Δ*) mutant cells fail to accumulate poly(A^+) RNA in the nucleus during heat shock. Further, Nab2 and Mlp1 form intranuclear foci upon heat shock whereas Mex67 retains a normal cellular distribution. Based on a direct physical interaction between Nab2 and Mex67-Mtr2, and their differential association in complexes from heat shocked cells, we propose a model whereby Slt2 and Mlp1 promote nuclear retention of non-hsp mRNPs by uncoupling the mRNA export receptor, Mex67, from specific mRNA binding proteins during heat shock. This mechanism allows selectivity for hsp mRNP export during stress and facilitates thermotolerance and rapid recovery.

Materials and Methods

Yeast strains, plasmids and growth

Yeast strains and plasmids used in this study are listed in Supplemental Table 4, respectively. Unless indicated otherwise, yeast cells were grown at 23°C in rich growth media (YPD) containing 1% yeast extract, 2% Bacto-peptone, and 2% glucose. For assaying thermotolerance and recovery from heat shock, cells were grown at 23°C to log phase, and then shifted to 52°C in a shaking water bath. At each time point, aliquots of 5 x 10^6 cells were taken and resuspended for serial dilution and spotting for plate growth at 23°C for 2-3 days. Site directed mutagenesis of *NAB2* was performed using pAC636 (*NAB2*) (Green et al., 2002)and CP436 (GST-Nab2) (Aitchison et al., 1996) as templates. To construct *T178A/S180A* mutants, PCR was performed with oligonucleotides 5'-ATGCAGACAGATGCTCCTGCAGCTCCAGCCCCCATATCAGCCTTTTCCGG-3',

and 5'-
CCGGAAAAGGCTGATATGGGGGCTGGAGCTGCAGGAGCATCTGTCTGCAT-3',
and to construct *T178E/S180E* mutants oligonucleotides 5'-
ATGCAGACAGATGCTCCTGCAGAGCCAGAGCCCATATCAGCCTTTTCCGG-3',
and 5'-
CCGGAAAAGGCTGATATGGGCTCTGGCTCTGCAGGAGCATCTGTCTGCAT-3'
were used. Resulting plasmids were sequenced to confirm mutations.

Immunoblot analysis of Nab2 phosphorylation

Cultures were grown to log phase in YPD at 30°C and shifted to 42°C for 1 hr, or to YPD containing 10% ethanol or 0.4M sodium chloride. Crude cell lysates were prepared by a protocol adapted from Yaffe and Schatz (1984.) 30 mg of cells were harvested, washed once with water, and lysed in 160 μl of 1.85M sodium hydroxide, and 7.4% β-mercaptoethanol on ice for 10 min. Protein was precipitated on ice for 10 min by addition of an equal volume of 50% trichloroacetic acid. Samples were centrifuged for 2 min at 15K relative centrifugal force, and pellets were washed 500 μl Tris base and resuspended in SDS sample buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with affinity-purified rabbit anti-Nab2 antibody (1:100,000) (Strawn et al., 2004), followed by HRP conjugated anti-rabbit secondary antibody. For phosphatase sensitivity assays, Nab2-TAP was immunoprecipitated from cells grown to early log phase in YPD, using IgG-coated sepharose beads. Beads were washed, resuspended in 1x λ phosphatase buffer, and

treated with either 100U of λ phosphatase, or 100U of λ phosphatase and phosphatase inhibitor cocktail (Calbiochem) for 30 min at 30°C.

Nab2-TAP purifications and Mass spectrometry

S. cerevisiae cells expressing Nab2-TAP were grown in YPD at 23ºC to an OD_{600} of 0.75. Samples for analysis of 23ºC growth were directly harvested by centrifugation, washed twice in 4°C H_2O, and once in resuspension buffer (20 mM HEPES pH=7.4, 1.2% polyvinylpyrrolidone, 1 mM DTT, 1 mM PMSF, 4μg/ml pepstatin A). Samples for analysis of 42ºC growth were obtained by shifting a 23°C culture rapidly to heat shock conditions by addition of 65°C YPD 1:1, and continued growth at 42°C for 1 hr before harvesting. Isolated cells pellets were injected into liquid nitrogen, and frozen cells were lysed in the solid phase by milling, using a planetary ball mill (as described in Oeffinger et al, 2007). Frozen cell lysate was thawed, and resuspended in extraction buffer (20 mM HEPES pH=7.4, 110 mM potassium acetate, 100 mM NaCl, 2 mM $MgCl_2$, 0.5% Triton X-100, 0.1% Tween 20, 1 mM PMSF, 4 μg/ml pepstatin A, 0.06 μl/ml Antifoam B Emulsion (Sigma)) and centrifuged 2 min, 900xg. Supernatant was incubated with rabbit IgG coated Dynabeads (Invitrogen) for 30 min at 4°C. Beads were collected magnetically, washed 3 times in extraction buffer, and 1 time in final wash buffer (100 mM ammonium acetate pH= 7.5, 0.2 mM $MgCl_2$, 0.05% Tween 20). Beads were then washed 3 times rapidly and 1 time for 5 min in final wash buffer lacking Tween 20. Protein was eluted by two washes with 500μl of 0.5 M NH_4OH, 5 mM EDTA, with shaking at room temperature. Elutions were pooled and lyophilized.

MudPIT mass spectrometry and phosphorylation site analysis were performed essentially described in (MacCoss et al., 2002). Briefly, purified protein complexes were digested in parallel with trypsin, elastase, and subtilysin. Peptides from these digests were acidified and loaded via pressure injection platform (New Objective, Woburn, MA) onto a 100 μm internal diameter, split-phase MudPIT (Washburn et al., 2001; Wolters et al., 2001); http://mitchison.med.harvard.edu/publications/IntJMSV219p245.pdf) column consisting of ~4 cm Aqua C18 reverse phase and ~4 cm Luna SCX (Phenomonex) fritted into an M520 filter union (IDEX, Oak Harbor, WA). After loading, this column was placed in-line with an 18 cm Jupiter (3 micron, 300A) 100 μm internal diameter, self-packed analytical column. Peptides were resolved using an Eksigent 1D+ HPLC system through a 16-hr MudPIT separation with eight 5 μl salt pulses (0 mM, 50 mM, 75 mM, 100 mM, 150 mM, 300 mM, 500 mM, and 1M ammonium acetate) followed by an organic gradient to resolve each eluted set of peptides. Tandem mass spectra were collected data-dependently using an LTQ equipped with a nanoelectrospray source. Spectra were searched with SEQUEST (Yates et al., 1995) considering possible phosphate modifications to serine, threonine, and tyrosine against an *S. cerevisiae* protein database containing a reversed-sequence decoy database. Results from these searches were filtered to a 5% peptide false discovery rate, collated, and accounted to a given analysis (23°C versus 42°C) using IDPicker (Ma et al., 2009). Spectra corresponding to potential sites of phosphorylation were confirmed by manual evaluation.

In vitro kinase assays

Yeast strains expressing TAP-tagged kinases were grown at 23°C to early log phase in YPD. Cells were harvested by centrifugation, washed once, and resuspended in 300μl in 1x TBT buffer (20 mM HEPES pH=7.4, 110 mM KOAc, 100 mM NaCl, 0.5% Triton X-100, 0.1% Tween, 0.18 mg/ml PMSF, 4 μg/ml pepstatin A, 0.06% antifoam B (Sigma)). Cells were lysed with 0.5 mm glass beads. The supernatant was isolated by centrifugation and incubated with IgG (Sigma)-coated magnetic beads (Invitrogen) for 30 min at 4ºC. Beads were washed 3x in TBT, and 1x in kinase buffer (20 mM HEPES pH=7.5, 20 mM β-glycerol phosphate, 10 mM $MgCl_2$, 10 mM PNPP, 100 μM Na_3VO_4, 2 mM DTT, 20 μM ATP). Beads were resuspended in 40μl kinase buffer containing 1 μg of recombinant Nab2 and 0.5 μl of γ-^{32}P ATP. Samples were incubated 30 min at 30ºC. The recombinant wild type Nab2, nab2-T178A/S180A, and nab2-T178E/S180E proteins were purified as described below. Supernatants were removed, and the beads were resuspended in 1x SDS-loading buffer. After heating at 100°C for 5 min, samples were separated by SDS-PAGE and the gel exposed to autoradiography film.

Microscopy

All images were acquired using a microscope (BX50; Olympus) with a UPlanF1 100× NA 1.30 oil immersion objective (Olympus) and a camera (CoolSNAP HQ; Photometrics). Within each experiment, all images were collected and scaled identically. Images were collected using Image-Pro Express 6.0 (Media Cybernetics) and processed with Photoshop 9.0 (Adobe). For *in situ* hybridization, yeast cells were grown in YPD to

early log phase at 23°C, and aliquots were shifted to 42°C for 45 min. Cells were fixed for 10 min and processed as previously described (Iovine et al., 1995; Wente et al., 1992). The digoxigenin-dUTP–labeled oligo d(T) probe was detected with fluorescein-labeled antidigoxigenin Fabs (1:50; Roche). DNA was stained with 0.1 μg/ml DAPI, and samples were mounted for imaging in 90% glycerol and 1 mg/ml *p*-phenylenediamine (Sigma-Aldrich), pH 8.0.

Assay for heat shock protein production

Yeast cells were grown to early log phase in synthetic complete media lacking methionine (SC-Met) at 23ºC, isolated by centrifugation and resuspended in 1ml SC-Methionine. For heat shock, 250μl of 59ºC SC-Met was added to 250μl cell suspension and placed at 42ºC for 15 min. For controls, 250μl of 23ºC SC-Met was added to 250μl cell suspension and maintained at 23ºC for 15 min. Cells were radiolabeled by the addition of 50 μCi ^{35}S-methionine to each sample, and incubated an additional 15 min before harvesting by centrifugation at 4ºC. Cells were washed 2x with 4ºC SC-Met, and lysed in 50μl SDS loading buffer at 100ºC for 5 min. Samples were separated by SDS-PAGE, and the resulting gel was dried and exposed to autoradiography film.

Poly(A$^+$) tail length determination

Cells were grown in YPD at 23ºC until early log phase, and shifted to 42ºC for 1 hr. Total RNA was end-labeled with ^{32}P-pCp and T4 RNA ligase. To digest non-

poly(A^+) RNA, the labeled RNA was treated simultaneously with RNases A/T1 and then ethanol precipitated. Resuspended RNA was resolved by denaturing urea-acrylamide gel electrophoresis and imaged using a phosphoimager.

In vitro binding assays

Soluble binding assays were performed using recombinant-purified Mex67-Mtr2 heterodimer, recombinant-purified Dbp5, recombinant-purified untagged Nab2 versions, and GST-Nab2 from bacterial lysate. The Dbp5 was purified as previously described (Alcazar-Roman et al., 2006; Tran et al., 2007). For the Mex67-Mtr2 purification, the coding regions for *MEX67* and *MTR2* were subcloned into a single pET-Duet1 (Novagen) plasmid such that Mtr2 would be N-terminally tagged with 6xHIS and Mex67 was untagged. This allowed co-expression of both proteins from a single vector. The heterodimer was expressed in Rosetta/DE3 by induction with IPTG. Cells were harvested and lysed by sonication under native conditions (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, including RNase, DNase, 1mM PMSF, 1x protease inhibitor tablet (ETDA-free, Roche)). Protein was purified using Ni-NTA resin per the manufacturer's recommendations (Qiagen) and eluted with 250 mM imidazole. Purified Mex67-Mtr2 was dialyzed into Buffer B (20 mM HEPES pH=7.5, 150 mM NaCl, 20% w/v glycerol).

For the soluble binding assays with wild type GST-Nab2 and glutathione resin, bacterial cells harboring Nab2-pGEX2TK were induced for expression of GST-Nab2 with IPTG. Cells were harvested, and total cell lysate was incubated with glutathione

resin, washed and subsequently incubated with the indicated Mex67-Mtr2 or Dbp5 proteins for 10 min at room temperature. The resin was then washed and bound proteins were eluted with 20 mM glutathione. 10% input (In) and 50% of bound (Bd) were resolved by gel electrophoresis and proteins were visualized by Coomassie staining.

For soluble binding assays with untagged recombinant Nab2 proteins, previously published methods were used to purify the proteins (Tran et al., 2007). Briefly, GST-Nab2, GST-nab2-T178A/S180A and GST-nab2-T178E/S180E were purified from bacterial lysates with glutathione Sepharose fast flow resin and the manufacturer's protocol (GE Healthcare, Piscataway, NJ). GST tag removal was performed by Thrombin protease cleavage and separation by affinity chromatography. Purified 6x-HIS-Mtr2/Mex67 was incubated with Ni^{2+} agarose beads at 4°C for 1 hr in MHL buffer (50 mM sodium phosphate pH=7.4, 150 mM $NaCl_2$, 10 mM imidazole, 10% glycerol) in batch. Resin was washed in MHL buffer, resuspended in MHL buffer, and split evenly in nine tubes. To each tube, 0.5μg, 0.25μg, and 0.1μg of purified wild type Nab2, nab2-T178A/S180A, or nab2-T178E/S180E protein was added, and incubated 1 hr at 4°C. Resin was washed three times in MHL buffer, and samples were collected in SDS sample buffer. Mex67 levels were detected by Coomassie stained SDS-PAGE, and Nab2 levels were detected by immunoblotting.

Results

Nab2 is phosphorylated under heat shock stress

The role of the essential mRNA binding protein Nab2 in hsp mRNA export has not been examined; yet, Nab2 is emerging as a key factor involved in coupling mRNA

processing and export events (Kelly et al., 2010; Roth et al., 2009; Viphakone et al., 2008). Of note, our previous work defined Nab2 as essential for bulk mRNA export and a physiological target for release from mRNPs during NPC translocation (Tran et al., 2007). Therefore, we examined whether Nab2 was altered during cell stress in *S. cerevisiae* cells. To this end, we focused on identifying Nab2 post-translational modifications that were dependent upon specific cellular stresses. Briefly, wild type *NAB2* and chromosomally tagged *NAB2-TAP* strains were grown to early logarithmic phase at 30°C in rich media and then exposed to multiple distinct stress conditions for 1 hr. These conditions included an osmolarity shift (to 0.4 M NaCl), addition of ethanol (to 10%), and heat shock (at 42°C). Upon completion of the stress condition shifts, whole cell lysates were prepared, resolved by gel electrophoresis, and analyzed by immunoblotting with anti-Nab2 antibodies. Neither the osmolarity nor ethanol shifts resulted in detectable changes in Nab2 electrophoretic mobility (Fig. 6A, lanes 3,4,7,8). In contrast, when cells were shifted to 42ºC, a second, slower migrating Nab2 band was observed for both untagged (Fig. 6A, lane 2) and TAP-tagged Nab2 (Fig. 6A, lane 6).

We speculated that the slower migrating Nab2 isoform resulted from phosphorylation. To test this hypothesis, endogenous Nab2-TAP was isolated by affinity purification from cell lysates following heat shock and phosphatase sensitivity was analyzed. Nab2-TAP was incubated with buffer alone (Fig. 6B, lane 1), λ phosphatase (Fig. 6B, lane 2), or λ phosphatase and a phosphatase inhibitor cocktail (Fig. 6B, lane 3). Samples were then resolved by gel electrophoresis and the presence of the slower migrating Nab2 band was visualized by immunoblotting as above. Strikingly, after incubation with λ phosphatase, Nab2 in the heat-shocked isolate was only present as a

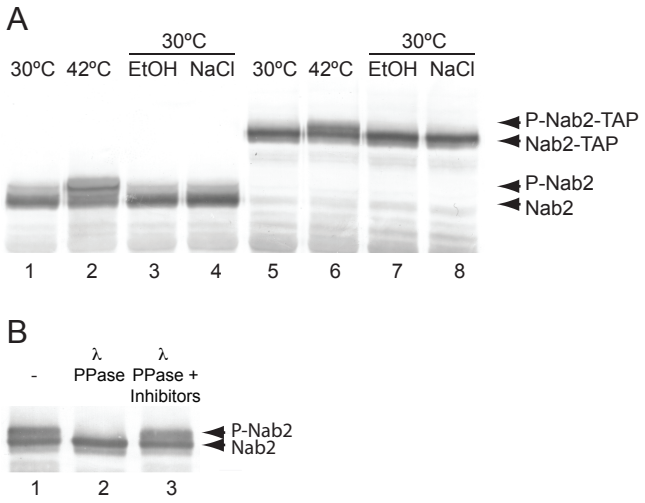


Figure 6: Nab2 is phosphorylated upon heat shock. (A) Immunoblotting for Nab2 (lane 1-4) or Nab2-TAP (lane 5-8) was conducted with cell lysates isolated after shifting cells from growth in YPD at 30ºC to 42ºC (lane 1, 2; and 5, 6) or shifting to growth in YPD with 10% EtOH (lanes 3 and 7) or YPD with 0.4M NaCl (lanes 4 and 8) for 1 hr. (B) Nab2-TAP was immunoprecipitated from cells in early log phase grown in YPD at 30ºC and shifted to 42ºC for 1 hr. Immunoprecipitates were treated with lambda phosphatase (lane 2), lambda phosphatase + phosphatase inhibitors (lane 3), or mock treatment (lane 1) for 30 min at 30ºC. Data provided by Elizabeth Tran.

phosphatase inhibitors. These results demonstrate that Nab2 is phosphorylated specifically in response to heat shock stress.

The Bck1/Slt2 pathway is required for heat shock-induced Nab2 phosphorylation

To identify the kinase responsible for Nab2 phosphorylation, we performed a screen using null (Δ) strains from the *S. cerevisiae* null collection. Based on the Protein Kinase Resource (http://www0.nih.go.jp/mirror/Kinases/pkr/pk_class/kinases_sc.html) and database annotations, 87 null mutants for the genes encoding non-essential protein kinases were selected and tested (Table 2). Each strain was grown in rich media at 23ºC, and shifted to 42ºC for 1 hr. Whole cell lysates were prepared and immunoblotting for endogenous Nab2 was conducted. Strikingly, only two of the tested mutants showed no Nab2 doublet formation after growth at 42ºC, *bck1*Δ and *slt2*Δ (Fig. 7A, lanes 4 and 6). Both Bck1 (the MAPKKK) and Slt2 (the MAPK) are components of the MAP kinase cell wall integrity pathway that is activated in response to heat shock and other cellular stresses (Chen and Thorner, 2007). Consistent with the redundant functions of Mkk1 and Mkk2 (MAPKKs), the Nab2 doublet was still observed in the respective single *mkk1*Δ and *mkk2*Δ mutants (data not shown). Examples of other kinase null mutants that showed maintained Nab2 doublet formation are shown in Fig. 7A (lanes 7-10). Taken together, these data implicate Slt2, the downstream kinase in the Bck1 pathway, as the *in vivo* kinase targeting Nab2 during heat shock stress.

Table 2: List of proteins tested in screen for Nab2 kinase.

Number	Standard Name	Systematic Name	Number	Standard Name	Systematic Name
1	Akl1	YBR059C	45	Pho85	YPL031C
2	Ark1	YNL020C	46	Pkh1	YDR490C
3	Atg1	YGL180W	47	Pkh2	YOL100W
4	Bck1	YJL095W	48	Pkh3	YDR466W
5	Bud1	YGR152C	49	Prr1	YKL116C
6	Cka2	YOR061W	50	Prr2	YDL214C
7	Cmk1	YFR014C	51	Psk1	YAL017W
8	Cmk2	YOL016C	52	Psk2	YOL045W
9	Dan1	YJR150C	53	Ptk1	YKL198C
10	Dbf2	YGR092W	54	Ptk2	YJR059W
11	Dbf2	YGR092W	55	Rck1	YGL158W
12	Elm1	YKL048C	56	Rck2	YLR248W
13	Env7	YPL236C	57	Rim15	YFL033C
14	Fmp48	YGR052W	58	Rtk1	YDL025C
15	Fpk1	YNR047W	59	Sak1	YER129W
16	Fus3	YBL016W	60	Skm1	YOL113W
17	Gcn2	YDR283C	61	Slt2	YHR030C
18	Gin4	YDR507C	62	Snf1	YDR477W
19	Hal5	YJL165C	63	Sps1	YDR523C
20	Hog1	YLR113W	64	Ssk2	YNR031C
21	Hrk1	YOR267C	65	Ssk22	YCR073C
22	Hsl1	YKL101W	66	Ssn3	YPL042C
23	Ime2	YJL106W	67	Ste11	YLR362W
24	Ire1	YHR079C	68	Ste20	YHL007C
25	Isr1	YPR106W	69	Ste7	YDL159W
26	Kcc4	YCL024W	70	Swe1	YJL187C
27	Kin1	YDR122W	71	Tda1	YMR291W
28	Kin2	YLR096W	72	Tos3	YGL179C
29	Kin3	YAR018C	73	Tpk1	YJL164C
30	Kin4	YOR233W	74	Tpk2	YPL203W
31	Kin82	YCR091W	75	Tpk3	YKL166C
32	Kka1	YJL094C	76	Twf1	YGR080W
33	Kns1	YLL019C	77	Vhs1	YDR247W
34	Ksp1	YHR082C	78	Vps15	YBR097W
35	Mck1	YNL307C	79	Yak1	YJL141C
36	Mds1	YMR139W	80	Yck2	YNL154C
37	Mek1	YOR351C	81	Yck3	YER123W
38	Mkk1	YOR231W	82	Ygk3	YOL128C
39	Mkk2	YPL140C	83	Ypk1	YKL126W
40	Mlp1	YKL161C	84	Ypk2	YMR104C
41	Mrk1	YDL079C	85	Uncharacterized ORF	YPL150W
42	Nnk1	YKL171W	86	Uncharacterized ORF	YBR028C
43	Npr1	YNL183C	87	Uncharacterized ORF	YNL141C
44	Pak1	YIL095W			

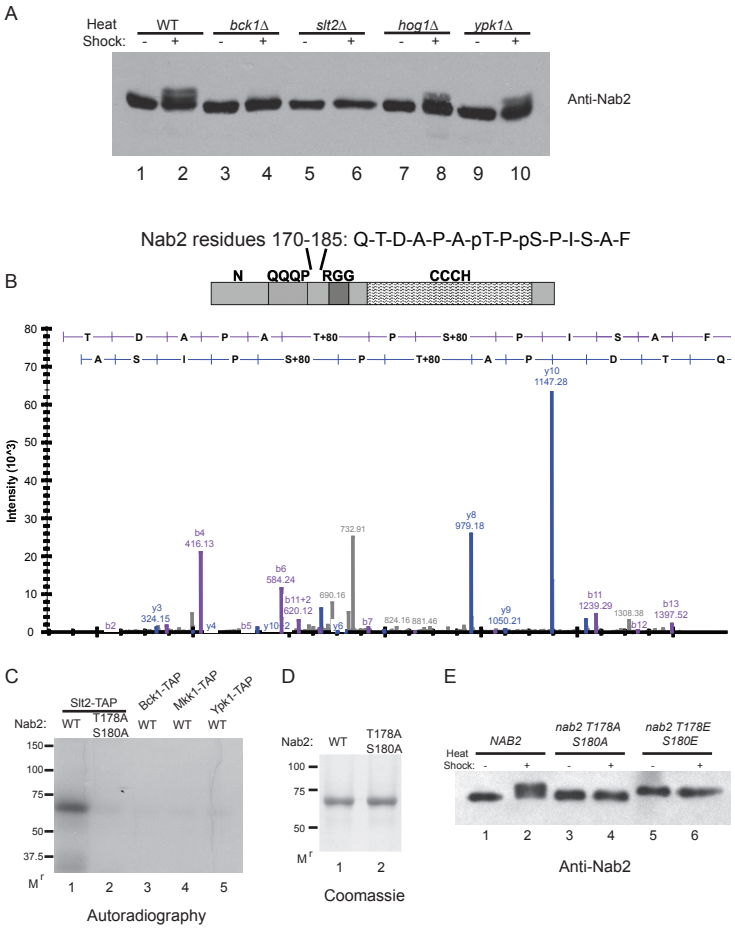


Figure 7: Nab2 phosphorylation occurs via the MAP kinase, Slt2, on residues 178 and 180. (A) *S. cerevisiae* kinase null mutants were screened by immunoblotting with anti-Nab2 antibodies for the Nab2 mobility shift. Cells were grown at 23ºC (-) (lanes 1,3,5,7,9), and shifted to 42ºC for 1 hr (lanes 2,4,6,8,10). (B) Diagram of Nab2 domain structure (Marfatia et al., 2003), with Nab2 LC/MS data for a peptide from residues 170 to 185 shown. Mass spectrometry was used to determine the phosphorylation sites on Nab2-TAP isolated from heat shocked cells. In the graph, mass spectrum intensity for the product b and y ions from the peptide are all shown. Overall, the mass spectrum indicates two sites are phosphorylated. T178 and S180 were identified because ions b4 (Q-T-D-A) and b6 (Q-T-D-A-P-A) have masses indicative of lack of phosphorylation on T171, and y3 (F-A-S) is indicative of lack of phosphorylation on S183, leaving T178 and S180 as the only possible phosphorylation targets. (C) *In vitro* kinase reactions were conducted with Slt2-TAP (lane 1, 2), Bck1-TAP (lane 3), Mkk1-TAP (lane 4), and Ypk1-TAP (lane 5) purified from *S. cerevisiae* on IgG-coated magnetic beads, and wild type recombinant Nab2 (lanes 1,3-5) or Nab2-T178A/S180A (lane 2), with gamma ^{32}P ATP for 30 min at 30ºC. Reactions were separated by a SDS-PAGE and exposed to autoradiography film. (D) Coomassie stained SDS-gel of Nab2 protein used in vitro kinase reactions in (C). (E) Immunoblotting for Nab2 was performed on lysates made from cells grown at either 23ºC (lanes 1,3,5), or shifted to 42ºC for 1 hr (lanes 2,4,6) in *nab2D* strains containing plasmids expressing either wild type *NAB2* (lane 1-2), *nab2-T178A/S180A* (lane 3-4), or *nab2-T178E/S180E* (lane 5-6). M^r = kDa

Slt2 directly phosphorylates Nab2 *in vitro*

To determine if Nab2 is a direct target of Slt2, *in vitro* kinase assays were conducted using purified, bacterially-expressed recombinant Nab2 and selected kinases from *S. cerevisiae*. Briefly, Slt2, Mkk1, Bck1, or Ypk1 were isolated from yeast cell lysates by TAP tag affinity isolation and subsequently incubated with purified recombinant Nab2 and γ-^{32}P-ATP for 30 min at 30°C. Following separation by gel electrophoresis, Nab2 phosphorylation was visualized by autoradiography. As shown in Fig. 7C (lane 1), the Slt2-TAP incubation resulted in Nab2 phosphorylation. However, the Mkk1-TAP, Bck1-TAP, and Ypk1-TAP did not promote ^{32}P incorporation (Fig. 7C, lanes 3, 4, and 5). This result confirms that Slt2 directly phosphorylates Nab2.

Nab2 is dually phosphorylated *in vivo* on threonine 178 and serine 180

As diagrammed in Fig. 7B, Nab2 is composed of distinct domains, which promote protein:protein interactions or RNA-binding activity *in vivo* (summarized in Marfatia et al., 2003): the C-terminal region contains seven zinc finger motifs which facilitate RNA-binding, the N-terminal region associates with Mlp1 to promote NPC docking, the central RGG motif is recognized by the karyopherin Kap104 for nuclear import and a central QQQP span is of unknown function. To understand the functional significance of Slt2-dependent modification, we determined the heat shock-induced *in vivo* phosphorylation sites on Nab2 using mass spectrometry (LC/MS). Briefly, Nab2-TAP protein was isolated from heat shocked yeast cells. The purified protein was digested with proteases, and the

resulting peptides were analyzed by LC/MS. Two sites of phosphorylation were detected by analyzing the resulting peptide fragments. These sites were threonine (T) 178 and serine (S) 180, both of which were identified on a single peptide spanning residues 170-185 (Fig. 7B). While this peptide has four possible sites of phosphorylation (T171, T178, S180 and S183), T178 and S180 were identified as the correct sites due to the presence of ions harboring the other potential sites (b4 (Q-T-D-A) and b6 (Q-T-D-A-P-A) for T171 and y3 (F-A-S) for S183), but that did not have the mass shift indicative of phosphorylation. Strikingly, the T178 and S180 residues lie in a region outside of the functionally mapped domains of Nab2, suggesting that this region might be important for previously unrecognized molecular interactions in the mRNA export pathway.

Both of the phosphorylated residues were in sequence regions that matched published predictions for MAP kinase consensus sites (as determined by the search algorithms available at http://scansite.mit.edu). Thus, we speculated that Slt2 catalyzes the modification of the T178 and S180 sites *in vivo*. We used site directed mutagenesis to construct a non-phosphorylatable Nab2 isoform (nab2-T178A/S180A). Recombinant Nab2-T178A/S180A was expressed and purified from bacteria, and subjected to *in vitro* kinase assays using isolated Slt2-TAP from yeast and ^{32}P-γATP (Figure 7C). In contrast to wild type Nab2 (Fig. 7C, lane 2), no ^{32}P was incorporated into the nab2-T178A/S180A protein. This change in phosphorylation status was not due to differences in protein levels (Fig. 7D). We conclude that Nab2 is phosphorylated at T178 and S180 by Slt2.

To verify that Nab2 T178 and S180 sites are phosphorylated *in vivo* during heat shock, yeast strains expressing non-phosphorylatable (T178A/S180A) or phosphomimetic (T178E/S180E) Nab2 isoforms were constructed. Briefly, yeast

LEU2/CEN plasmids harboring *nab2-T187A/S180A* or *nab2-T178E/S180E* mutant alleles were transformed into a *nab2Δ* yeast strain maintained by a wild type *NAB2*/*URA3/CEN* plasmid. The resulting strains were streaked on media containing 5-fluoroorotic acid, yielding viable strains with only the *nab2-T178A/S180A* or *nab2-T178E/S180E* genes. Testing for cell growth across a range of temperatures revealed that T178 and S180 were not required for general cell viability (data not shown, Fig. 11). Wild type and mutant strains were grown at 23ºC, shifted to 42ºC, and whole cell lysates were generated and tested by immunoblotting with anti-Nab2 antibodies. Unlike wild type Nab2, the nab2-T178A/S180A protein did not show a shift in migration following growth at 42ºC. This finding further supports the conclusion that T178/S180 are *in vivo* phosphorylation sites targeted during heat shock (Fig. 7E, lanes 3 and 4). In addition, the phosphomimetic nab2-T178E/S180E protein exhibited reduced gel migration at both 23°C and 42°C (Fig. 7E, lanes 5 and 6), most likely reflecting the overall change in the Nab2 protein isoelectric charge. Taken together, these studies demonstrate that Nab2 is phosphorylated during heat shock at T178/S180 by the MAP kinase Slt2.

Poly(A^+) mRNA does not accumulate in the nucleus upon heat shock in *slt2Δ* cells

When *S. cerevisiae* cells undergo heat shock, most poly(A^+) mRNA is retained in the nucleus while hsp mRNAs are exported to the cytoplasm. Since heat shock dependent post-translational modification of an essential mRNA export factor might provide a means for differential mRNA transport, we hypothesized that Slt2 plays a critical role in

this process. To test this idea, *in situ* hybridization experiments were conducted to localize poly(A^+) mRNA in wild type and *slt2Δ* cells at both the permissive temperature and following heat shock at 42˚C. Strikingly, whereas wild type cells showed strong nuclear accumulation of poly(A^+) RNA indicative of normal heat shock induced retention of non-hsp mRNA, *slt2Δ* cells did not show nuclear accumulation of poly(A^+) mRNA (Fig. 8A). In fact, the poly(A^+) mRNA localization in the *slt2Δ* cells was diffuse and throughout the cell consistent with a lack of nuclear poly(A^+) mRNA retention. As *nab2* mutants have polyadenylation defects (Kelly et al., 2010), we conducted control experiments to analyze total poly(A^+) tail length in *slt2Δ* cells. No defects were detected (Fig. 9). This indicates that the loss of Slt2 does not lead to altered polyadenylation, which could have indirectly impacted poly(A^+) localization. Thus, the lack of nuclear poly(A^+) mRNA accumulation during heat shock in *slt2Δ* cells reflects a change in non-hsp mRNA retention.

To test whether Nab2 phosphorylation is the sole Slt2 target responsible for poly(A^+) nuclear mRNA accumulation during heat shock, poly(A^+) mRNA localization in the *nab2-T178A/S180A* mutant cells was analyzed by *in situ* hybridization. The *nab2-T178A/S180A* mutant cells still showed nuclear accumulation of poly(A^+) mRNA at 42ºC (Fig. 8B), indicating that Nab2 phosphorylation is not sufficient to mediate non-hsp mRNA retention during heat shock. Consistent with this result, the *nab2-T178E/S180E* mutant showed no growth defect and did not result in nuclear poly(A^+) accumulation under non-heat shock conditions (data not shown). Taken together, this indicates that there are additional unidentified Slt2 kinase targets that affect the mRNA export mechanism

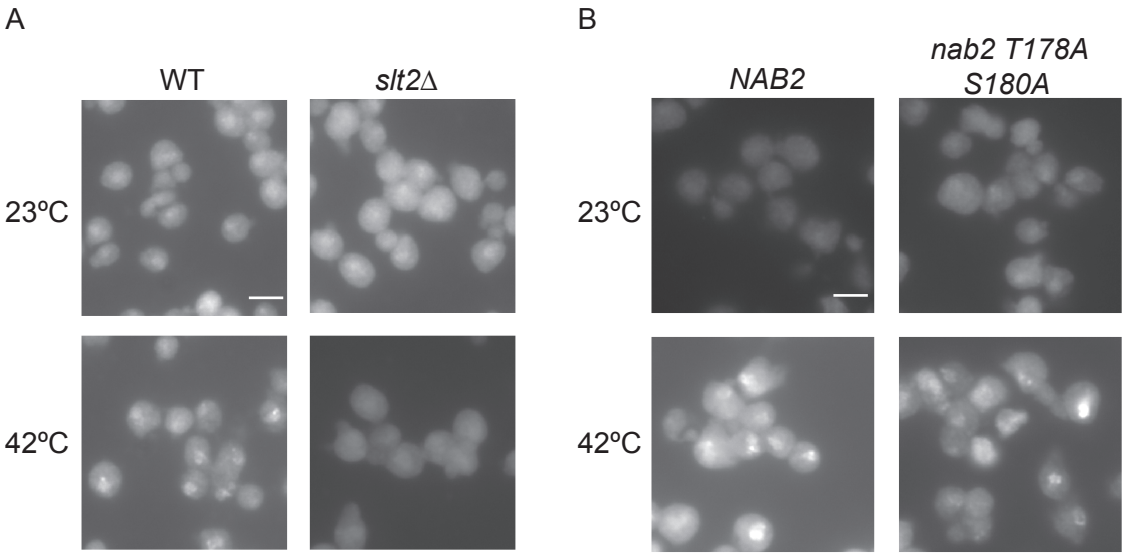


Figure 8: Poly(A^+) mRNA does not accumulate in the nucleus of heat shocked *slt2*Δ cells. *In situ* hybridization using an oligo d(T) probe was performed on WT and *slt2*Δ cells (A) and wild type and *nab2-T178A/S180A* cells (B) grown to early log phase in YPD at 23ºC (top rows) or after shift to 42ºC (bottom rows) for 45 min. Bar = 3 μm.

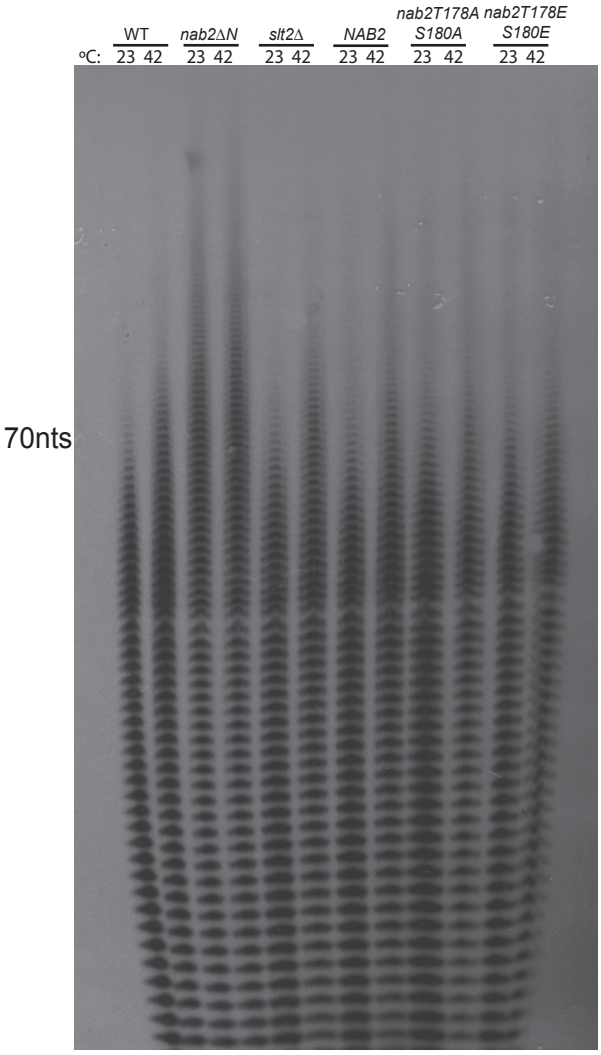


Figure 9: Poly(A^+) tail length is unaffected in *slt2*Δ, *nab2-T178A/S180A*, and *nab2-T178E/S180E*. Total RNA was isolated from wild type, *slt2Δ, nab- T178A/S180A*, and *nab2-T178E/S180E* cells grown at 23ºC or after a 1 hr shift to 42ºC. RNA was end-labeled with ^{32}pCp and T4 RNA ligase, and digested with RNase A and RNase T1 to remove non-poly(A^+) tracks. Resulting stretches of poly(A^+) were then resolved by denaturing urea-polyacrylamide gel electrophoresis and visualized by autoradiography. The position of the 70 nucleotide (nt) typical poly(A^+) tail length is marked on the left. Data provided by Luciano Apponi, Anita Corbett laboratory, Emory University.

Heat shock proteins are produced under stress conditions in *bck1* and *slt2* mutants

In addition to inhibiting bulk non-hsp mRNA export upon heat shock, Nab2 phosphorylation and the Slt2 MAPK pathway could also actively facilitate the export of hsp mRNA. To test this possibility, we shifted *bck1*Δ and *slt2*Δ cells to 42ºC for 15 min to induce heat shock stress and the cells were pulse-labeled with ^{35}S-methionine to monitor heat shock protein synthesis. After an additional 15 min at 42ºC, total cell lysates were prepared, separated by gel electrophoresis and radiolabeled proteins were visualized by autoradiography. Both wild type and *nup42Δ* mutant cells were included as controls. The *nup42Δ* mutant has a documented defect in hsp mRNA export and subsequent heat shock protein production (Saavedra et al., 1996; Saavedra et al., 1997; Vainberg et al., 2000). Consistent with published reports, heat shock proteins were efficiently synthesized in wild type cells but absent in *nup42Δ* cells (Fig. 10, lanes 2 and 4*)*. Both *slt2Δ* and *bck1Δ* strains showed robust synthesis of heat shock proteins (Fig. 10, lanes 6, and 8), similar to wild type cells. Thus, Slt2 is not required for synthesis of heat shock proteins and likely plays no role in facilitating export of hsp mRNA. Furthermore, these studies demonstrate that loss of Slt2 does not result in global defects in transcriptional upregulation during stress.

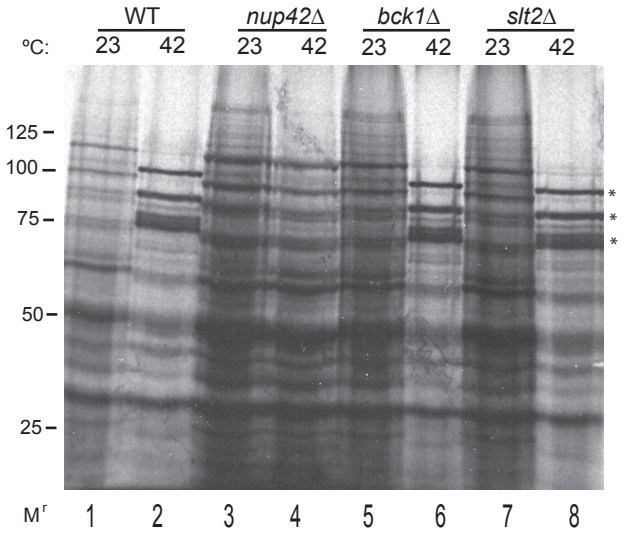


Figure 10: Production of heat shock proteins is not affected in *slt2*Δ or *bck1*Δ cells. Wild type (lane 1-2), *nup42*Δ (lane 3-4), *bck1*Δ (lane 5-6), and *slt2*Δ (lane 7-8) cells were grown at 23ºC, shifted to 42ºC for 15 min, and labeled with ^{35}S methionine for an additional 15 min. Cell lysates were separated by SDS-PAGE and proteins were visualized by autoradiography. Asterisks at the right indicate proteins induced upon heat shock (Hsp104, Hsp82, and Hsp70s respectively). M^r = kDa.

Nab2 phosphorylation is required for the recovery of a *nup42Δ* mutant from heat shock

Our results are consistent with a role for Slt2 in retention of non-hsp mRNAs during heat shock. Moreover, others have reported that Slt2 is required for cellular response to stress (Martin et al., 1993; Millson et al., 2005; Truman et al., 2007). To understand the function of Nab2 phosphorylation in cell stress, we speculated that incorporation of *nab2* mutants in strains with deficient hsp mRNA export would result in growth and thermotolerance defects. To test this hypothesis, we asked if *nab2-T178A/S180A* and *nab2-T178E/S180E* mutants alone or in combination with *nup42Δ* mutant exhibited enhanced temperature sensitivity under normal growth conditions (Fig. 11). To assay for defects in heat shock recovery, the mutant cells were exposed to extreme heat shock (52˚C) (Nwaka et al., 1996) for 5-20 min time periods and then assayed for growth recovery at 23°C by serial dilution. Strikingly, the *nup42Δ nab2-T178A/S180A* strain showed a substantial reduction in viability after heat shock as compared to the wild type and single mutant strains (Fig. 11). Importantly, the *nup42Δ nab2-T178E/S180E* double mutant did not have diminished recovery from heat shock. Taken together, this indicates that Nab2 phosphorylation is needed for optimal cellular heat shock survival. Thus, we speculate that Nab2 phosphorylation is linked to non-hsp RNA retention in the nucleus and this promotes recovery following return to normal growth conditions. Further, *nab2-T178A/S180A*, *nab2-T178E/S180E* were also tested in combination with *gle1-4*, *dbp5-2*, and *mex67-5*, mutants in the bulk mRNA export pathway (Figures 12, 13, and 14), as well as *mlp1*Δ (Figure 15). None of these mutants

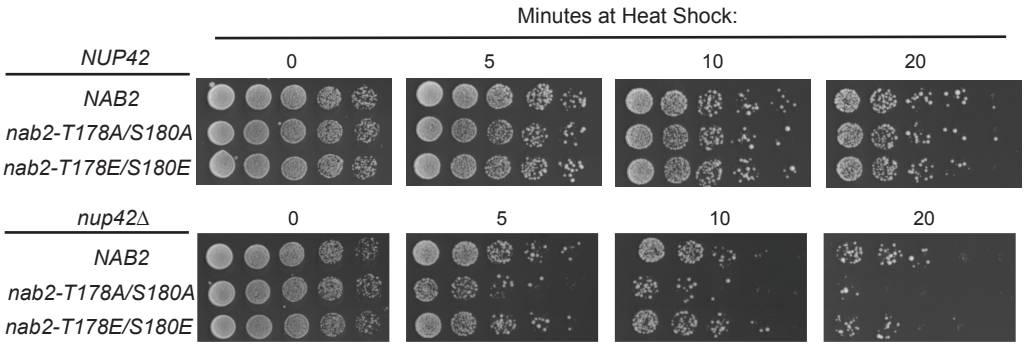


Figure 11: Nab2 phosphorylation is required for efficient recovery from heat shock. Cells were grown at 23°C prior to shifting to 52°C for the times indicated (0,5,10, and 20 min). Aliquots of same cell number were then serially diluted and plated for growth at 23°C on YPD for 2.5 days.

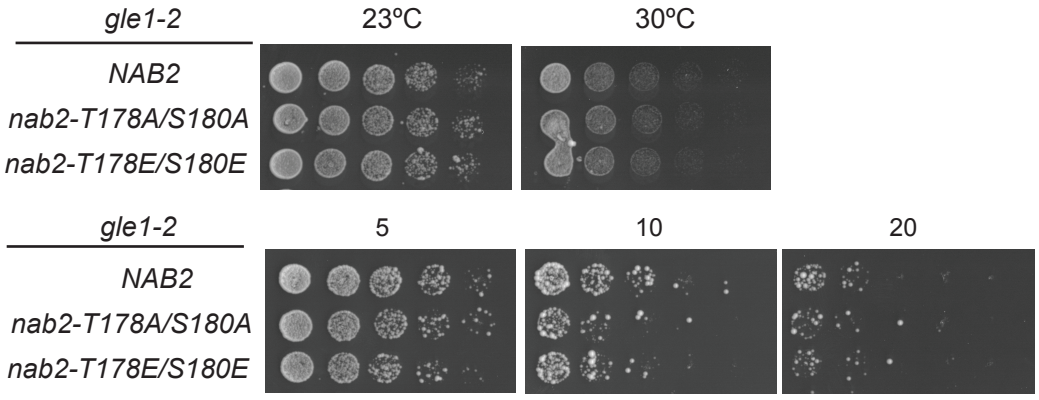


Figure 12: Nab2 phosphorylation mutants do not affect growth in *gle-2*. Cells were grown at 23°C, aliquots of same cell number were serially diluted, and grown for 2.5 days at the indicated temperature (upper panel). Cells were grown at 23°C prior to shifting to 52°C for the times indicated (5,10, and 20 min). Aliquots of same cell number were then serially diluted and plated for growth at 23°C on YPD for 2.5 days (lower panel). The 23°C from the upper panel is the zero time point for the lower panel.

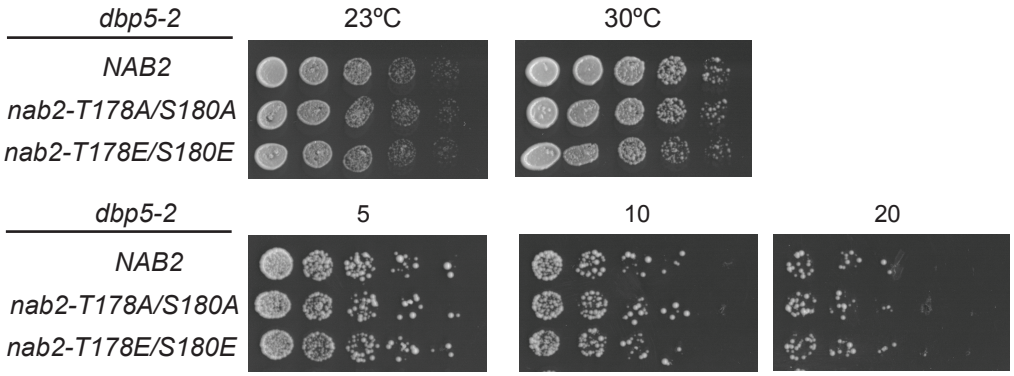


Figure 13: Nab2 phosphorylation mutants do not affect growth in *dbp5-2*. Cells were grown at 23°C, aliquots of same cell number were serially diluted, and grown for 2.5 days at the indicated temperature (upper panel). Cells were grown at 23°C prior to shifting to 52°C for the times indicated (5,10, and 20 min). Aliquots of same cell number were then serially diluted and plated for growth at 23°C on YPD for 2.5 days (lower panel). The 23°C from the upper panel is the zero time point for the lower panel.

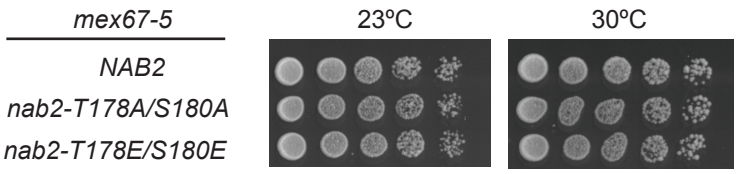


Figure 14: Nab2 phosphorylation mutants do not affect growth in *mex67-5*. Cells were grown at 23°C, aliquots of same cell number were serially diluted, and grown for 2.5 days at the indicated temperature.

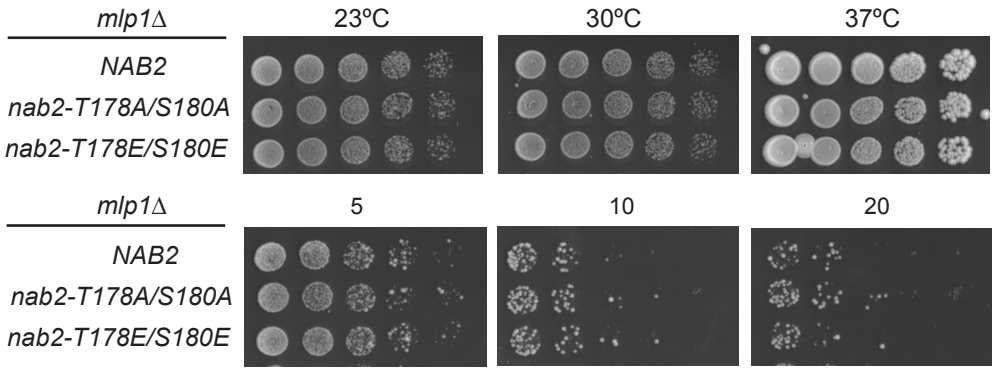


Figure 15: Nab2 phosphorylation mutants do not affect growth in *mlp1*Δ. Cells were grown at 23°C, aliquots of same cell number were serially diluted, and grown for 2.5 days at the indicated temperature (upper panel). Cells were grown at 23°C prior to shifting to 52°C for the times indicated (5,10, and 20 min). Aliquots of same cell number were then serially diluted and plated for growth at 23°C on YPD for 2.5 days (lower panel). The 23°C from the upper panel is the zero time point for the lower panel.

affected growth of *nab2-T178A/S180A*, or *nab2-T178E/S180E* either after heat shock, or when grown at constant temperature. Notably, *nab2-T178A/S180A* and *nab2-T178E/S180E mex67-5* could not be tested after heat shock in this assay, as a *mex67-5* strain alone is dead after the 5 minutes at 52°C.

Nab2, Mlp1 and Yra1 form heat shock-dependent intranuclear foci

Nab2 shuttles between the nucleus and cytoplasm in a RNA polymerase II-dependent manner (Duncan et al., 2000; Marfatia et al., 2003). Thus, Nab2 cellular localization and/or shuttling activity might be altered by phosphorylation and upon heat shock. To test this possibility, we analyzed Nab2 localization under normal and heat shock conditions by live cell imaging using a mCherry or green fluorescent protein (GFP)-tagged Nab2. Strikingly, Nab2-mCherry or Nab2-GFP accumulated in nuclear foci upon heat shock (Fig. 16A and 16B, respectively, left panels). These foci were not due to the epitope tag as Nab2 foci were also observed by indirect immunofluorescence microscopy with anti-Nab2 antibodies (data not shown). Due to the documented Nab2-Mlp1 interaction and the Mlp1 role in retention of aberrant mRNAs during normal cellular growth, we next asked if Mlp1, Mlp2 and/or Nups localize to nuclear foci upon heat shock. Whereas the nuclear basket Nup60-GFP and centrally localized Nup49-GFP remained associated with the nuclear envelope (Figure 17), both Mlp1 and Mlp2 formed nuclear foci upon heat shock (Fig. 16A, right panel, Fig. 17). Indeed, by co-localization analysis, Nab2-mCherry and Mlp1-GFP co-localized in intranuclear foci during heat shock (Fig. 16A).

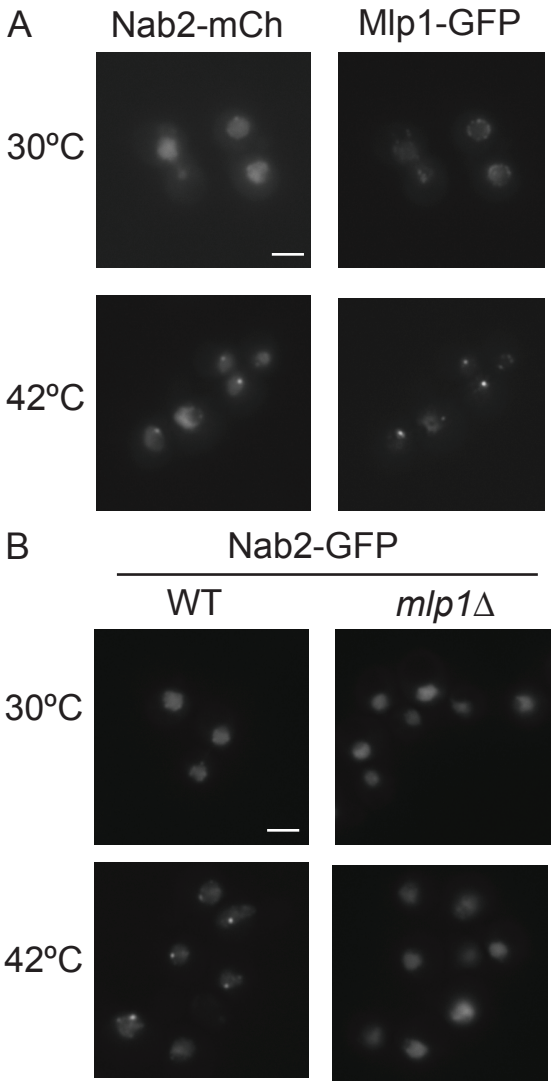


Figure 16: Nab2 forms *MLP1* dependent foci during heat shock. Cells were grown to early log phase at 23ºC and shifted to 42ºC for one hr. In wild type cells (left panels), Nab2-GFP shifts from total nuclear localization at 30°C to nuclear foci at 42°C. (A) Nab2-mCherry forms intranuclear foci at 42ºC that overlap with foci formed by Yra1-GFP. (B) In *mlp1*Δ cells (right panel), Nab2-GFP foci do not form at 42°C. Bar = 3µm. Data provided by Elizabeth Tran.

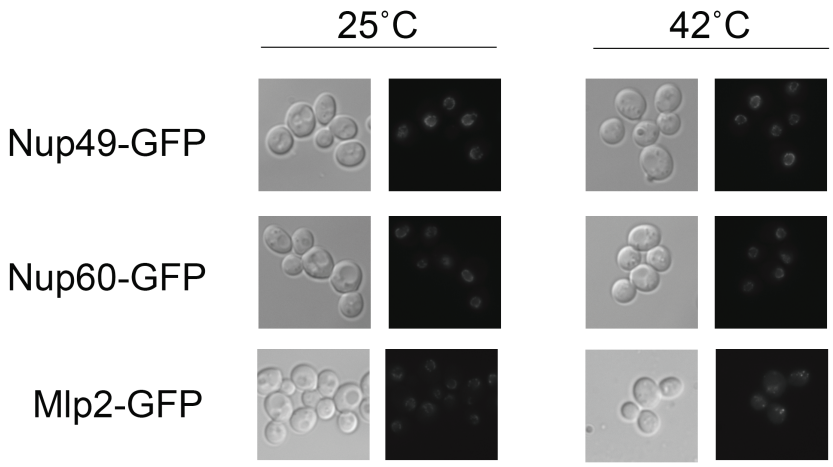


Figure 17: Differential localization of NPC-associated proteins during heat shock. Mlp2-GFP is localized in discrete intranuclear foci after shifting to growth at 42°C. Nup49-GFP and Nup60-GFP are localized to the nuclear rim during growth at 25°C and 42°C. Left column shows direct GFP fluorescence, and right column shows DIC. Data provided by Elizabeth Tran.

To determine if Mlp1 and/or Mlp2 are required for Nab2 foci formation, localization was analyzed in different null mutant strains. Loss of Mlp1 clearly inhibited foci formation (Fig. 16B, right panel). However, Nab2-GFP foci were not dependent on the Mlp2, Nup60, or the Mlp1-associated spindle assembly checkpoint protein Mad1 (De Souza et al., 2009; Scott et al., 2005) (Fig. 18). Thus, Mlp1 is necessary for formation of heat shock-induced Nab2 foci.

To test whether Slt2 promotes formation of Nab2 foci, a *NAB2-mCherry slt2Δ* strain was tested. Nab2-mCherry foci were observed in the *slt2Δ* cells after heat shock (Fig. 19A). To determine if foci formation is linked to Nab2 phosphorylation, immunoblotting for Nab2 was conducted in different null mutants. The Nab2 doublet was present in the *mlp1Δ* mutant after heat shock, as well as in the *nup42Δ* mutant (Fig. 19B). Lack of *GFD1,* which encodes another Nab2 binding protein (Suntharalingam et al., 2004), also had no apparent effect on phosphorylation. Thus, Nab2 phosphorylation and foci formation are independent. As controls, we tested if *mlp1*Δ cells had defects in either hsp mRNA export or nuclear retention of bulk poly(A^+) mRNA during heat shock. Following a shift to heat shock conditions, the *mlp1Δ* cells showed normal production of hsp proteins and accumulated nuclear poly(A^+) mRNA (Fig. 20, and data not shown). Taken together, these studies demonstrate that Slt2 and Mlp1 mediate distinct molecular interactions during heat shock.

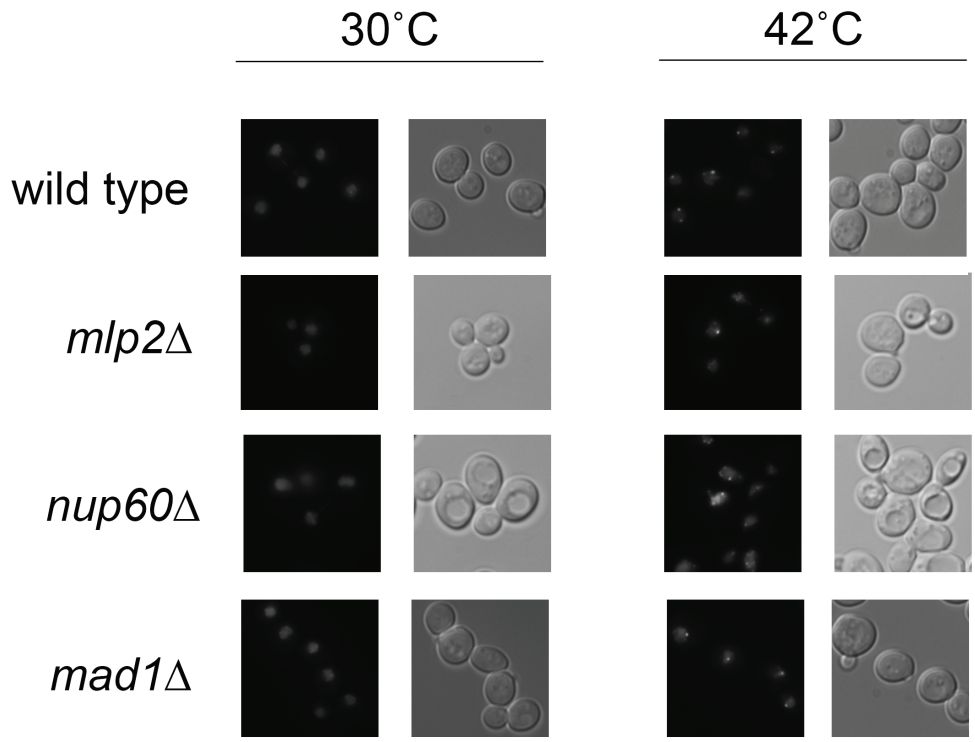


Figure 18: Formation of intranuclear Nab2-GFP foci is not dependent on other NPC-associated proteins. Yeast null mutants expressing Nab2-GFP were shifted from growth at 30°C to 42°C for 1 hr. Intranuclear foci formation was evaluated by direct fluorescence microscopy. Data provided by Elizabeth Tran.

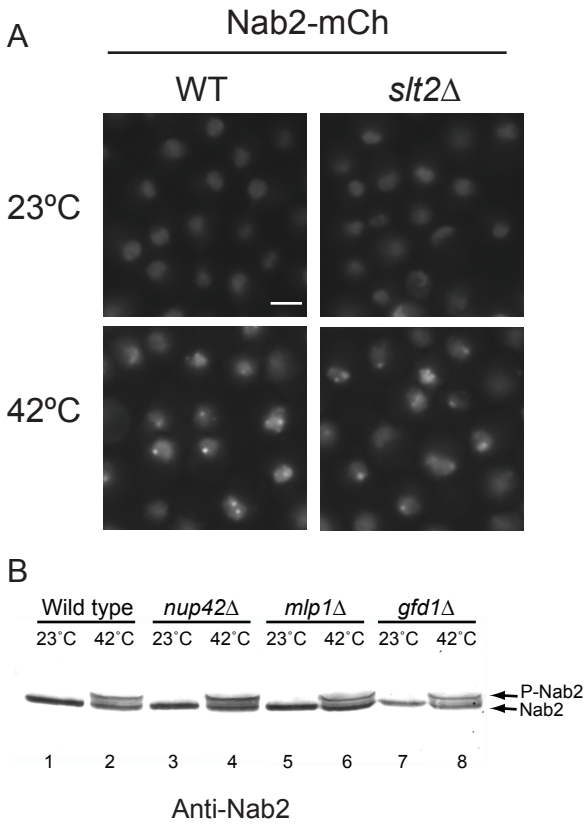


Figure 19: During heat shock, Nab2 phosphorylation and intranuclear foci formation are independent events. (A) Nab2-mCherry forms intranuclear foci in wild type (left panels) and *slt2D* (right panels) cells shifted to growth at 42°C for 1 hr. (B) Immunoblotting for Nab2 in lysates from yeast strains shows heat shocked induced (42°C for 1 hr) mobility shift in wild type (lane 1-2), *nup42*Δ (lane 3-4), *mlp1*Δ (lane 5-6), and *gfd1*Δ (lane 7-8) cells. Bar = 3μm. Data in B provided by Beth Tran.

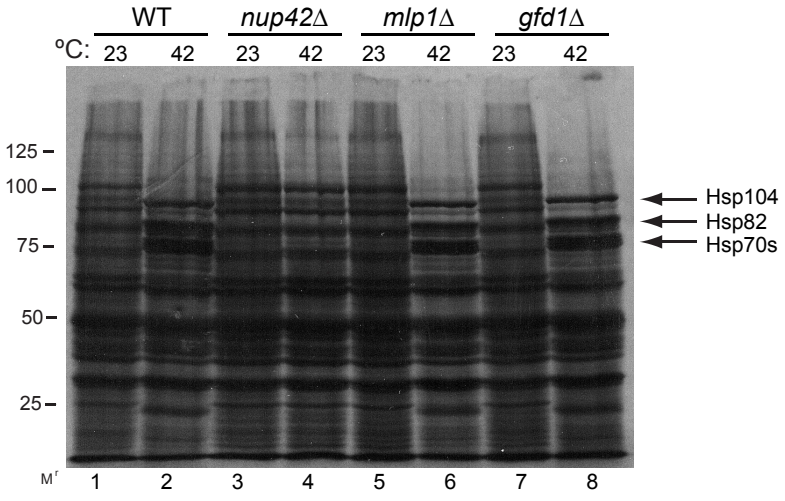


Figure 20: Production of heat shock proteins is not affected in *mlp1*Δ cells. Wild type (lanes 1-2), *nup42*Δ (lane 3-4), *mlp1*Δ (lane 5-6), and *gfd1*Δ (lane 7-8) cells were grown at 23°C, shifted to 42°C for 15 min, and labeled with ^{35}S methionine for an additional 15 min. Cell lysates were separated by SDS-PAGE and proteins were visualized by autoradiography. Asterisks at the right indicate proteins induced upon heat shock (Hsp104, Hsp82, and Hsp70s respectively.) Data provided by Elizabeth Tran.

Nab2-Mex67 interactions are uncoupled during heat shock

Because Slt2 is needed for nuclear accumulation of non-hsp mRNA, we speculated that stress signaling might alter interactions between Nab2 and other essential mRNA export factors during heat shock. A recent proteomic study reported Nab2 association with members of the TREX complex, including Yra1 and Mex67 (Batisse et al., 2009). To determine if heat shock uncouples Nab2 from TREX, first, the subcellular localizations of Yra1-GFP and Mex67-GFP were determined. Whereas Yra1-GFP localized to nuclear foci during heat shock (Fig. 21A), Mex67-GFP remained associated with the nuclear rim (Fig. 21B). Importantly, Yra1-GFP and Nab2-mCherry foci co-localized (Fig. 21A). Second, we employed biochemical approaches to analyze changes in Nab2 complexes from normal and heat shock induced cells. Nab2-TAP protein complexes were isolated from yeast cells grown at 23°C or following a shift to growth at 42°C for one hr. Cells were lysed using a liquid nitrogen grinding method that preserves native mRNP interactions (Batisse et al., 2009; Oeffinger et al., 2007). Purified complexes were then analyzed by multidimensional protein identification technology (MudPIT) mass spectrometry to determine the protein composition. Included in Table 3 are all co-purifying proteins with documented mRNP or NPC links. The co-purifying proteins at 23°C largely parallel that reported by Batisse et al., (2009), with the exception of Sub2. We did not detect Sub2 in our Nab2-TAP isolation, potentially due to strain, isolation, or detection differences. Interestingly, we found that the Nab2-TAP complexes varied greatly in composition when isolated from heat shocked versus non-heat shocked cells. To quantify each co-purifying protein, total spectral counts of each were taken, and normalized to the total spectral counts for Nab2 in the same sample (Table 3). The

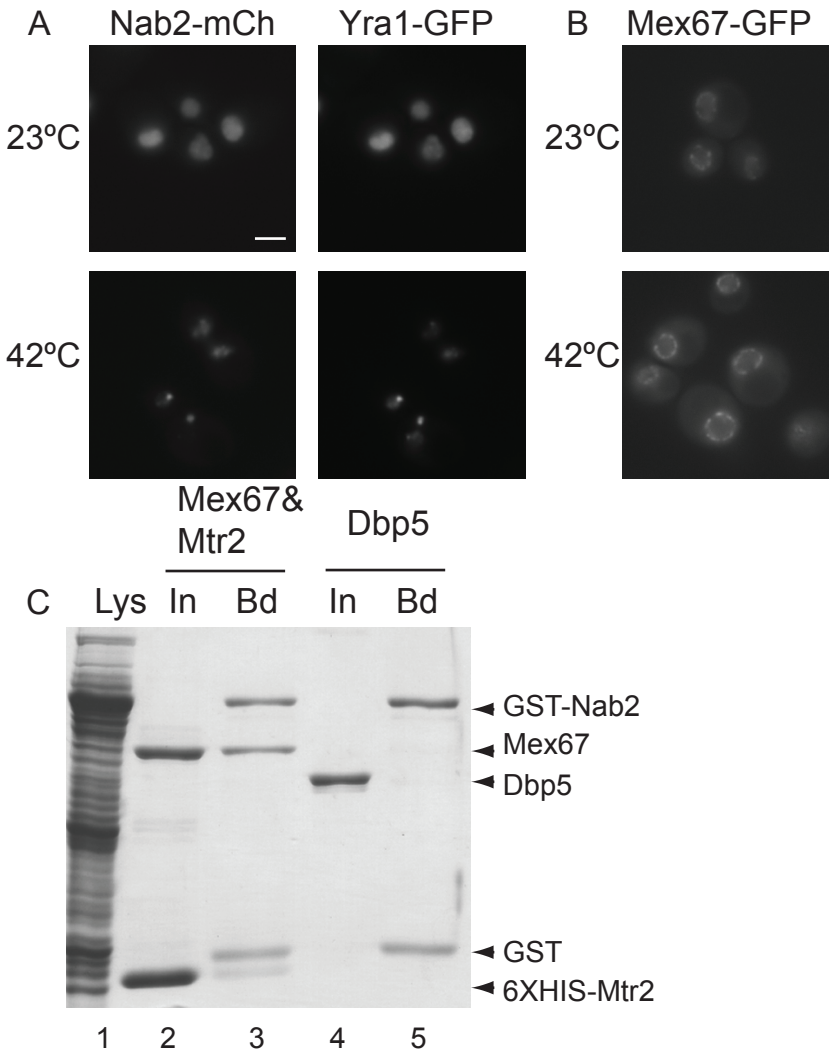


Figure 21: Nab2-Mex67 directly interact and are not co-localized during heat shock. (A) Cells co-expressing Nab2-mCherry and Yra1-GFP were tested for formation of heat shock-induced intranuclear foci. Nab2-mCherry and Yra1-GFP co-localize in foci at 42°C. Bar = 3 μm. (B) Mex67-GFP localization does not change during heat shock, and remains nuclear envelope localized. (C) Nab2 and Mex67-Mtr2 directly interact *in vitro*. Soluble binding assays with glutathione resin were conducted with purified recombinant Mex67-Mtr2 (lane 2-3), or Dbp5 (lane 4-5) with lysate (Lys) from bacterial cells expressing GST-Nab2 (lane 1). Samples were separated by SDS-PAGE and proteins visualized by Coomassie staining. Input (In) and bound (Bd) fractions are shown. GST alone as a proteolytic product from GST-Nab2 was observed in lanes 3 and 5. Data in B and C provided by Beth Tran.

Table 3: Mass spectrometry analysis of Nab2-TAP complexes isolated from normal and heat shocked cells

A	B	C	D	E	F
Protein	**Raw Spectral Count 23°C**	**Raw Spectral Count 42°C**	**Normalized Spectral Count 23°C**	**Normalized Spectral Count 42°C**	**Relative 42°C/23°C level (E/D)**
Nab2	561	257	100	100	1
Mlp1	113	349	20.1	135.8	6.8
Mlp2	77	158	13.7	61.5	4.5
Yra1	56	60	10	23.4	2.3
Pab1	218	209	38.9	81.3	2.0
Mex67	45	10	8.0	3.9	0.5
Gfd1	40	51	7.1	19.8	2.8
Stm1	16	15	2.9	5.8	2
Tho2	9	6	1.6	2.3	1.4
Nsp1	185	17	33	6.6	0.2
Pom152	116	13	20.7	5.0	0.24
Nup170	94	3	16.7	1.2	0.07
Nup159	76	9	13.5	3.5	0.26
Nup188	72	6	12.8	2.3	0.18
Nup145	83	3	14.8	1.2	0.08
Nup60	76	7	13.5	2.7	0.2
Nup133	76	0	13.5	0	0
Nup2	66	3	11.7	1.2	0.10
Nic96	67	4	11.9	1.6	0.13

Nup157	63	5	11.2	2.0	0.18
Nup192	63	3	11.2	1.2	0.10
Nup85	53	1	9.5	0.9	0.09
Nup82	50	7	8.9	2.7	0.30
Sac3	53	1	9.5	0.4	0.04
Nup120	47	0	7.7	0	0
Nup1	29	3	5.2	1.2	0.23
Gle2	30	0	5.4	0	0
Nup59	59	0	2.6	0	0
Pom34	16	2	2.9	0.8	0.28
Nup53	14	1	2.5	0.4	0.16

resulting ratio reflected the relative level of each protein to Nab2-TAP (Table 3, row F). At 23°C, NPC associated proteins were the largest class found to be co-purifying with Nab2-TAP. Strikingly, the relative amounts of all the detected NPC proteins decreased when Nab2-TAP was isolated from heat shocked cells (with a value < 0 in column F.) Moreover, the Mex67 levels were also decreased after heat shock. In parallel, after heat shock, increased amounts of Mlp1, Mlp2, and Yra1 were isolated in Nab2-TAP complexes (those with a value > 0 in column F). Overall, the biochemical analysis was consistent with the distinct localization patterns of these factors upon heat shock. We conclude that Mex67 association with Nab2-complexes is inhibited during heat shock stress.

Current models predict that Yra1 acts as an adaptor protein for Mex67 recruitment to mature mRNPs (Strasser and Hurt, 2000). However, Yra1 is constitutively nuclear and thus, is not exported to the cytoplasm with a Mex67-bound mRNP. In contrast, Nab2 shuttles to the cytoplasm in an export dependent manner (Marfatia et al., 2003; Suntharalingam et al., 2004). Based on the differential association of Mex67 with Nab2 complexes, we speculated that Nab2 might be a previously unrecognized adaptor for Mex67-Mtr2. To test for direct physical interactions, *in vitro* binding assays were conducted with purified recombinant Mex67-Mtr2 heterodimer as previously described (Yao et al., 2007). Briefly, glutathione-S-transferase (GST)-tagged Nab2 was expressed in bacteria (Fig. 21C, lane 1) and lysate was bound to glutathione resin. Purified Mex67-Mtr2, or as a control the DEAD-box protein Dbp5, was applied to the GST-Nab2 resin. Following washes, bound proteins were eluted with glutathione and separated by gel electrophoresis. Coomassie staining showed isolation of Mex67-Mtr2 with the GST-Nab2

(Fig. 21C, lane 3), whereas Dbp5 was not (Fig. 21C, lane 5). Previous studies have documented that Mex67 does not bind to GST or glutathione resin non-specifically (Terry and Wente, 2007), and the Mex67-Mtr2 interaction with Nab2 was also verified by an alternative binding assay described below. The direct in vitro binding of recombinant Mex67-Mtr2 to Nab2 suggests that Nab2 could serve as a direct adaptor for Mex67-Mtr2 recruitment to mRNPs.

Taking into account both the in vitro Nab2 interaction with Mex67-Mtr2 and the decreased in vivo Mex67-Mtr2 association with Nab2 complexes isolated from heat-shocked cells, we speculated that Nab2 phosphorylation during heat shock stress might impact Nab2 interaction with Mex67-Mtr2. To test this, in vitro soluble binding assays were conducted with purified recombinant nab2-T178E/S180E protein and Mex67-Mtr2. For these assays, Mex67-Mtr2 was immobilized on $Ni2^{+}$ agarose beads via the 6xHIS epitope tag on Mtr2. Decreasing amounts of untagged wild type Nab2, nab2-T178A/S180A, or nab2-T178E/S180E protein were incubated with the Mex67-Mtr2 beads, and following washes the bound proteins were eluted and levels analyzed by immunoblotting. No difference in Mex67-Mtr2 interaction was detected between the Nab2 proteins (Fig. 22). Thus, Nab2 phosphorylation is potentially not sufficient to inhibit Mex67 recruitment to Nab2 complexes during heat shock, and other potential Slt2 targets and Mex67 interaction partners are playing roles. This model is illustrated in Fig. 24, where Mex67:Mtr2 is recruited to bulk mRNA by Nab2, and Yra1, and mRNPs are successfully exported from the nucleus. During heat shock, however, Slt2 phosphorylates Nab2, and one or more unidentified target proteins, resulting in nuclear mRNA retention, potentially mediated by Mlp1. Heat shock mRNAs, on the other hand,

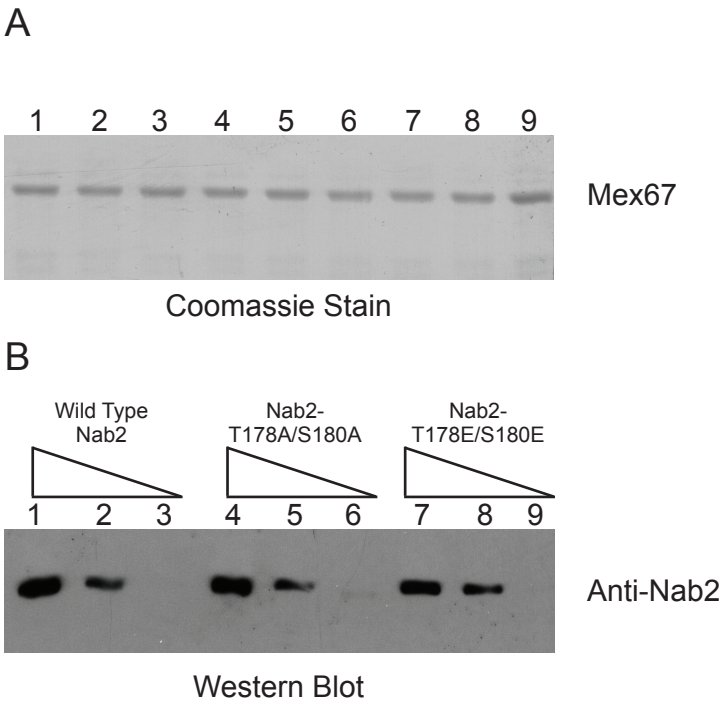


Figure 22: The nab2-T178E/S180E protein binds Mex67-Mtr2. 6x-HIS-Mex67/Mtr2 was bound in batch to Ni^{2+} agarose beads at 4°C, and divided equally into nine tubes. Purified recombinant wild type Nab2 (lanes 1-3), nab2-T178A/S180A (lanes 4-6), or nab2-T178E/S180E (lanes 7-9) protein was added to individual tubes in decreasing amounts of 0.5µg, 0.25µg, and 0.1µg. Samples were incubated for 1 hr at 4°C. Beads were washed, and boiled in SDS sample buffer to elute bound proteins. Mex67 was detected by Coomassie stained SDS-PAGE (A), and Nab2 was detected by immunoblotting (B).

still recruit Mex67:Mtr2, through a currently unknown adaptor protein, and are thus successfully exported.

Search for other mRNA-associated Slt2 phosphorylation targets

Due to the observation that a strain harboring a non-phosphorylatable version of Nab2 (nab2 T178A/S180A) does not phenocopy a strain lacking SLT2, the Nab2 kinase, we predicted that there is another phosphorylation target, or targets, of Slt2 that are necessary for the retention of non-heat shock mRNA. To address this, I used a bioinformatics based approach to try to identify another Slt2 target(s). Using the Saccharomyces Genome Database (SGD- http://yeastgenome.org/), and gene ontology (GO) annotation I searched for ORFs whose encoded proteins have a molecular function designated as 'RNA binding' and whose cellular component is designated as 'nucleus.' This search yielded 137 different proteins. In order to focus the search on proteins that may have a role in mRNA export, I removed proteins from the list that were involved in ribosome biogenesis, nucleolar functions, and splicing. While mutants defective in splicing do not export their non-spliced transcripts, I predicted that the bulk mRNA retention seen during heat shock is not likely do to this effect, because only a small percentage of the S. cerevisiae genome is spliced. Removal of these proteins resulted in a list 53 remaining proteins, not including, Nab2. Using the phosphorylation prediction program Scansite (http://scansite.mit.edu/), I searched each of these 53 proteins for potential MAP kinase phosphorylation sites that passed as 'medium stringency.' Of the two detected Nab2 phosphorylation sites, one passed as high stringency, and the other as

medium stringency on Scansite. This resulted in a final set of eleven proteins that met all criteria: 1-RNA-binding, 2-Nuclear, 3-not involved in ribosome biogenesis or splicing, and 4-have potential predicted MAP kinase phosphorylation sites. Of these eleven, one is directly involved in mRNA export (the mRNA export receptor Mex67), one in tRNA nuclear export (Los1), one in mRNA localization (Hek2), and the remaining eight in different stps of mRNA metabolism, including decapping, polyadenylation. Nine of these eleven proteins exist as TAP or GFP fusions in one of the genome wide S. cerevisiae libraries, and these nine were tested for possible Slt2 phosphorylation during heat shock by growing the strains at 23ºC, shifting to heat shock conditions of 42ºC for one hour, preparing cell lysates, western blotting, and looking for band of decreased mobility in the 42ºC samples. Along with the nine proteins that passed through the screening process, Yra1 was also included in this analysis, due to its localization in Nab2 foci during heat shock. Of these tested proteins, none showed altered migration patterns after shift to 42ºC. Nine are present as single bands, and Yra1 migrates as two distinct bands, but this is seen in both 23ºC and 42ºC samples (Figure 23), indicating that none of the tested proteins are likely to be phosphorylated by Slt2. While this search did not yield a second potential Slt2 target involved in mRNA retention, one is still likely to exist based on the differing phenotypes of nab2-T178A/S180A, and slt2Δ. The failure of this method to detect such a protein is likely due to it not being exhaustive, and could also potentially be due to a lack of a band shift of a phosphorylated protein.

A.

Gene	Biological Function	Strain Tested
Yra1	mRNA Export	GFP
Hek1	Telomere Maintenance	GFP
Los1	tRNA Export	TAP
Dcp2	Decapping	TAP
Hrp1	3' End Procressing	TAP
Mex67	mRNA Export	TAP
Nrd1	Transcription Termination	TAP
Pti1	3' End Procressing	TAP
Fip1	3' End Procressing	TAP
Cth1	Transcription	None
Pta1	3' End Procressing	None

B.

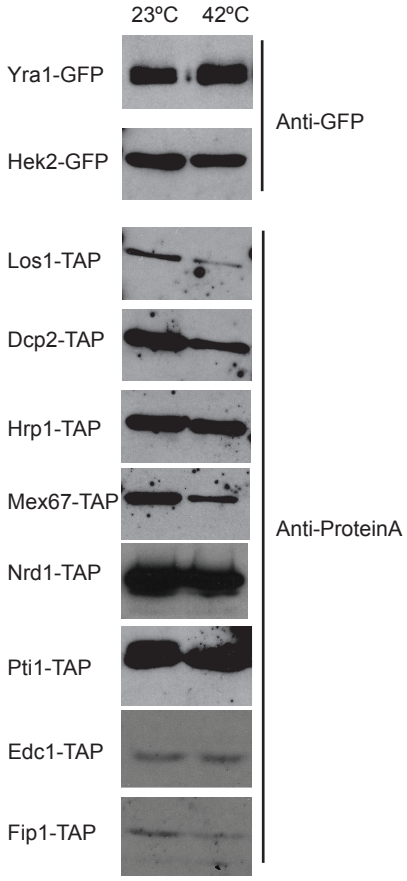


Figure 23: Search for putative mRNA-binding Slt2 phosphorylation targets. Potential Slt2 phosphorylation targets with a role in mRNA retention were determined using the advanced search method on the *Saccharomyces* genome database, and the phosphorylation prediction program at scansite.mit.edu. Proteins annotated as both locating to the nucleus, and RNA-binding, with putative phosphorylation sites are listed in (A). Proteins from (A) with strains available in either the genome wide GFP or TAP tag collection were screened by immunobloting (B) for decreased mobility, indicative of phosphorylation, after heat shock at 42ºC.

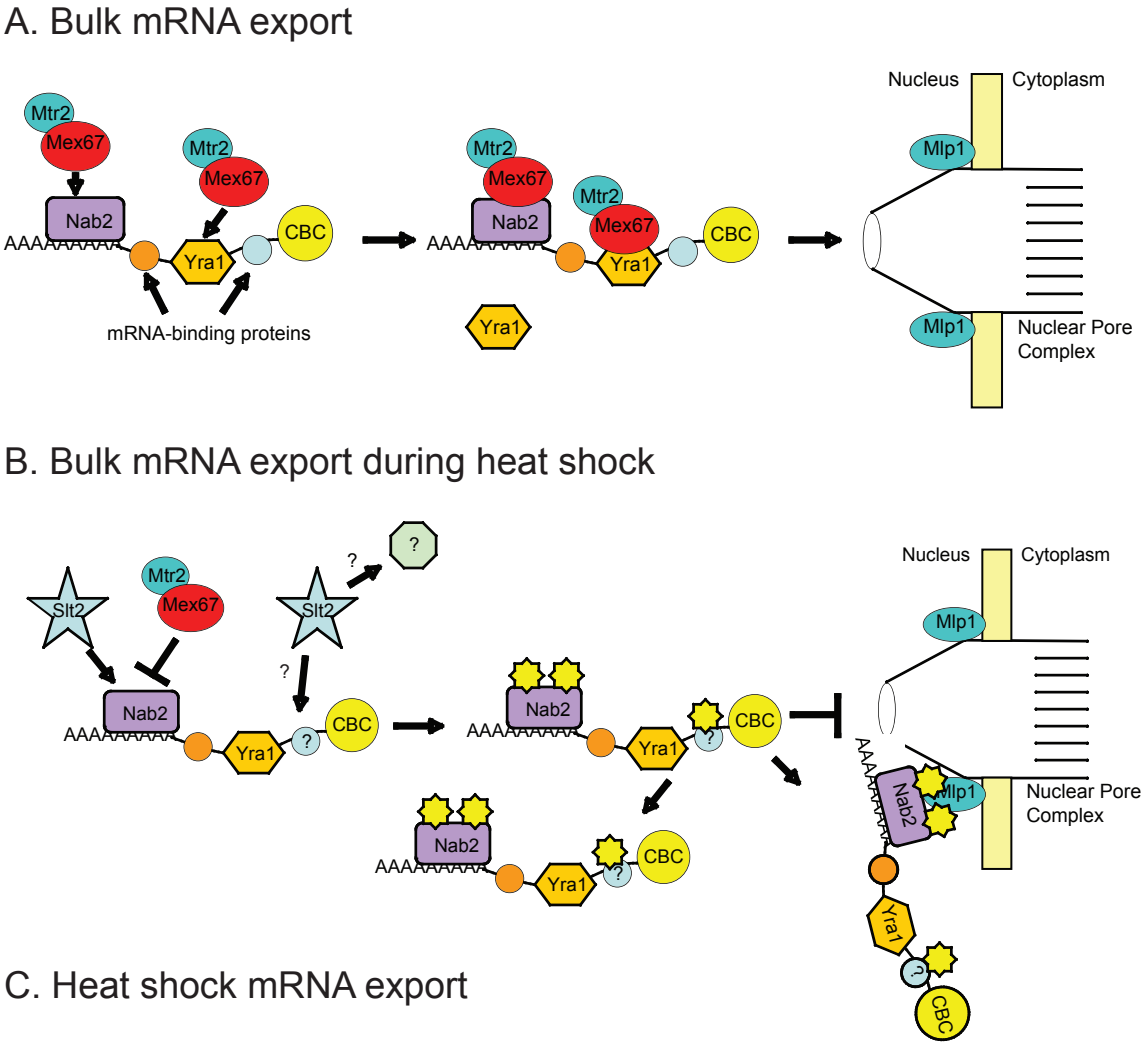


Figure 24: Model for mRNA export during heat shock and non-heat shock conditions. (A) During non-heat shock conditions, Mex67:Mtr2 is recruited bulk mRNA via adaptor proteins such as Nab2, and Yra1, and exported from the nucleus. (B) During heat shock, Nab2 is dually phosphorylated by Slt2, Mex67 recruitment is inhibited, and non-heat shock mRNA are retained in the nucleus, some bound to Mlp1. Slt2 is also phosphorylating currently unidentified targets, which also play a role in retention. (C) During heat shock, heat shock mRNA are selectively exported in a Mex67-dependent manner, likely by an uncharacterized adaptor protein.

Screen for other mRNA-binding proteins that localize to Nab2-containing foci during heat shock

Having determined that Mex67, but not Yra1, is uncoupled from Nab2 during heat shock, we next tested a panel of GFP tagged versions of mRNA-binding proteins, and processing factors to determine if any other proteins aggregate into foci during heat shock. Proteins tested included the mRNA-binding proteins, Gbp2, Hrp1, Npl3, Pab1, Pub1, Sub2, and Tho2, and the mRNA processing factors Pap1, Pcf11, Rat1 and Rrp6. Interestingly, none of these proteins relocalized to foci during heat shock (data not shown). This could be due to either lack of maturation of the transcripts in the foci (e.g., unspliced), or to the transcripts being specifically enriched in Nab2 and Yra1, versus other mRNA-binding proteins. Taken together with the lack of Mex67 recruitment to Nab2 containing transcripts, this indicates that Nab2, and Yra1 are preferentially associated with retained transcripts during heat shock.

Discussion

Defining the mechanism for regulated mRNA export during stress is critical to understanding cellular adaptation and survival. Here we have uncovered a functional correlation during heat shock stress between changes in Nab2 phosphorylation, localization, and association with Mex67 and Mlp1. We identify the cell wall integrity MAP kinase Slt2 signaling pathway as key in the mRNA export mechanism during heat shock. Two specific heat shock-induced Slt2 phosphorylation sites in Nab2 are needed for thermotolerance and stress recovery in a *nup42Δ* mutant. As *slt2Δ* cells do not accumulate nuclear poly(A^+) RNA upon heat shock but remain competent for hsp mRNA

export and poly(A^+) tail length maintenance, we conclude that Slt2 is required for nuclear non-hsp mRNA retention during stress. Further, based on decreased levels of Mex67 and Nups in Nab2 complexes and independent relocalization and association of Nab2 and Yra1 with Mlp1-dependent nuclear foci, we conclude that at least two independent mechanisms control the differential export of non-hsp and hsp mRNA during heat shock stress: regulated recruitment of Mex67-Mtr2 to mRNA binding proteins and sequestering of mRNA binding proteins in Mlp1 intranuclear foci.

This work directly addresses the long-standing question of whether an active mechanism exists for promoting hsp mRNA transport or if non-hsp mRNAs are selectively retained. Prior work has shown that both hsp and non-hsp mRNA export require the Mex67 mRNA export receptor as well as the direct mediators of mRNA transport, Dbp5, Gle1 and Nup159 (summarized in Rollenhagen et al., 2004; Rollenhagen et al., 2007; reviewed in Iglesias and Stutz, 2008). Moreover, hsp mRNA export is not dependent on Npl3 or Yra1, and others have shown that Npl3 re-localizes to the cytoplasm during heat shock to uncouple from hsp mRNA export (Krebber et al., 1999). We now document a critical role for Nab2, Slt2, and Mlp1 in heat shock-induced retention of bulk poly(A^+) mRNA and reinforce the hypothesis that the hsp mRNA export mechanism is distinct from the non-hsp global poly(A^+) mRNA export mechanism.

We propose a model wherein activated Slt2 promotes the retention of non-heat shock mRNAs through modulation of mRNA-binding proteins during heat shock stress. Under normal growth conditions, Mex67-Mtr2 is recruited to nascent mRNAs via the concerted action of Yra1 and Nab2. The Mex67-Mtr2/Nab2-bound mRNP is released from the site of synthesis and then docks at the nuclear face of the NPC. Interactions

between Nab2 and Mlp1 at the NPC provide a quality control step to monitor the proper mRNP composition of a given mRNA prior to pore translocation. During heat shock, the MAP kinase Slt2 is activated by the cell wall integrity phosphorylation cascade. This activation results in phosphorylation of Nab2 and possibly other components of the mRNA export machinery. Other signaling pathways are also likely triggered to independently impact mRNA export factors.

We further speculate that sequestering of Nab2 and Yra1 in Mlp1-dependent intranuclear foci, in combination with Slt2 signaling, inhibits recruitment of Mex67-Mtr2 to non-hsp mRNPs and diminishes export of such mRNPs. As such, under heat shock conditions, Nab2 complexes are not found associated with NPC proteins, as they are not undergoing NPC targeting or nuclear export. Overall, this mechanism might allow Mex67-Mtr2 to associate predominantly with hsp mRNAs through an uncharacterized, Nab2-independent mechanism. Such selective mRNA transport and retention could provide a means to tightly control gene expression: by coordinating transcription and export of hsp mRNAs necessary for cellular stress responses at the same time as retaining housekeeping (non-hsp) mRNAs needed for cell growth and recovery after the stress.

Our observations also highlight future questions to address regarding the mechanism for regulated mRNA transport during cellular stress. First, how is Mex67-Mtr2 recruited to hsp mRNAs? To date, none of the characterized mRNA adaptor proteins for Mex67 (Yra1, Npl3 and Nab2) are required for hsp mRNA export. There might be an uncharacterized mRNA binding protein that serves as a Mex67-Mtr2 adaptor protein on hsp mRNAs. Alternatively, Mex67-Mtr2 might directly bind and recognize hsp mRNAs. Indeed, Mex67-Mtr2 can associate directly with a stem loop structure in the

5S rRNA component of the large ribosomal subunit (Yao et al., 2007). The hypothesis that a specific RNA sequence mediates hsp mRNA export was first suggested by Cole and coworkers, based on the discovery of 5' and 3' UTR elements in the *SSA4* hsp mRNA that are both necessary and sufficient for directing export during heat shock (Saavedra et al., 1996).

Another intriguing question encompasses whether and how differential mRNA transport is employed during other non-heat shock growth conditions. Although our studies demonstrate key steps required for hsp versus non-hsp mRNAs transport during stress, it is possible that similar mechanisms exist for controlling transport of specific gene transcripts under other conditions. This could include other environmental stresses, such as salt and ethanol shock (Izawa et al., 2008), as well as during changes in cellular programs such as mating, cellular differentiation, and the cell cycle. Based on work showing Npl3 dynamics are influenced by the Sky1 kinase and Glc7 phosphatase (Gilbert and Guthrie, 2004), there is tremendous potential for the role of post-translational modifications of mRNA binding proteins in cell regulation. There are also interesting parallels to consider between the mechanisms for controlling mRNA export during heat shock and how viruses pirate the cellular mRNA export machinery. Indeed, Nup42 (Rip1) was first identified based on its role in facilitating Rev export in yeast cells (Stutz et al., 1995).

Alternatively, Nab2-Mlp1 foci might play an as-of-yet uncharacterized role that is distinct from mRNA export. Because Mlp1 and Mlp2 have been linked to downregulation of transcription (Vinciguerra et al., 2005), these foci could influence gene expression during stress. Future characterization of the Mlp1-Nab2-Yra1 foci is required

to decipher these possibilities. Work will also be needed to reveal the mechanism for intranuclear foci formation and Mlp1 relocalization. As the vertebrate Tpr is targeted by ERK2 (Vomastek et al., 2008), Mlp1 itself is also potentially targeted by post-translation modification during cellular stress. We predict that multiple signaling events impinge on both mRNP and NPC components to coordinate selective transport events.

Recent studies demonstrate that the MAP kinase Ste20 plays a key role in formation of cytoplasmic RNP foci called stress granules (Yoon et al., 2010) It is intriguing to consider that the Nab2-Yra1-Mlp1 intranuclear foci that form during heat shock stress parallel the role of cytoplasmic stress granules. As such, they could store non-hsp mRNAs or mRNA binding proteins during times of stress to allow rapid recovery after stress. Overall, this places the post-translational modifications and subcellular localization of mRNA binding proteins as key elements for controlling selective nuclear mRNA export and retention.

Table 4: *S. cerevisiae* strains used in this study

Strain	Genotype	Reference/Source
BY4742	Matα *his3Δ1 leu2Δ0 ura3Δ1 lys2Δ1*	Research Genetics
Nab2-TAP	*NAB2-TAP:HIS5* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
bck1Δ	*BCK1::KAN* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Winzeler et al., 1999)
slt2Δ	*SLT2::KAN* Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ1*	(Winzeler et al., 1999)
hog1Δ	*HOG1::KAN* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Winzeler et al., 1999)
ypk1Δ	*YPK1::KAN* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Winzeler et al., 1999)
SWY4307	*NAB2::HIS3* Matα *his3Δ1 leu2Δ0 trp1Δ ura3Δ1* p(AC636)	(Marfatia et al., 2003)
SWY4308	*NAB2::HIS3* Matα *his3Δ1 leu2Δ0 trp1Δ ura3Δ1* p(SW3579)	This study
SWY4309	*NAB2::HIS3* Matα *his3Δ1 leu2Δ0 trp1Δ ura3Δ1* p(SW3580)	This study
SLT2-TAP	*SLT2-TAP:HIS5*(*Sp*) Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
BCK1-TAP	*BCK1-TAP:HIS5*(*Sp*) Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
MKK1-TAP	*MKK1-TAP:HIS5*(*Sp*) Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
YPK1-TAP	*YPK1- TAP:HIS5*(*Sp*) Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
nup42Δ	*NUP42::KAN* Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ1*	(Winzeler et al., 1999)
SWY4493	*NUP42::KAN NAB2::HIS3 Matα his3Δ1 leu2Δ0 trp1Δ ura3Δ1* p(AC636)	This study
SWY4494	*NUP42::KAN NAB2::HIS3 Matα his3Δ1 leu2Δ0 trp1Δ ura3Δ1 p*(SW3579)	This study
SWY4495	*NUP42::KAN NAB2::HIS3 Matα his3Δ1 leu2Δ0 trp1Δ ura3Δ1* p(SW3580)	This study
SWY4435	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pAC 636 *(NAB2 CEN LEU2)*	This study
SWY4436	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pSW3579 *(nab2-T178A/S180A CEN LEU2)*	This study

SWY4437	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY4438	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + p(*nab2C437S/CEN/LEU2*)	This study
SWY4439	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY4440	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY4441	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pSW3579 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY4442	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP LYS ADE2 ADE3* p(*nab2C437S/CEN/LEU2*)	This study
SWY4443	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pAC 636 (*NAB2 CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY4444	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pSW3579 (*nab2-T178A/S180A CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY4445	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pSW3580 (*nab2-T178E/S180E CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY4446	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* p(*nab2-C437S*) p(*mex67-5 TRP1*)	This study
SWY4481	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY4482	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1*+ pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY4483	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY4484	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* p(*nab2C437S/CEN/LEU2*)	This study
SWY4552	*NAB2-mCherry:HygB YRA1-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 ura3Δ1*	This study
SWY4225	*NAB2-mCherry:HygB MLP1-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 ura3Δ1*	This study
SWY4034	*NAB2-GFP:HIS5*(*Sp) MLP1::KAN* Matα *his3Δ1 leu2Δ0 ura3Δ1*	This study
MEX67-GFP	*MEX67-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Huh et al., 2003)
SWY4225	*NAB2-mCherry:HygB MLP1-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	This study
SWY4251	*NAB2-mCherry:HygB MLP1-GFP:HIS5*(*Sp) SLT2::KAN* Mata *leu2Δ0 ura3Δ1*	This study

mlp1Δ	*MLP1::KAN* Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ1*	(Winzeler et al., 1999)
gfd1Δ	*GFD1::KAN* Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ1*	(Winzeler et al., 1999)
pAC1152	*NAB2::HIS3 p(nab2ΔN LEU2)* Mata *leu2 ura3*	(Marfatia et al., 2003)
YRA1-GFP	*YRA1-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Huh et al., 2003)
HEK2-GFP	*HEK2-GFP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Huh et al., 2003)
LOS1-TAP	*LOS1-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
DCP2-TAP	*DCP2-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
HRP1-TAP	*HRP1-TAP:HIS5*(*Sp* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
MEX67-TAP	*MEX67-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
NRD1-TAP	*NRD1-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
PTI1-TAP	*PTI1-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
EDC1-TAP	*EDC1-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)
FIP1-TAP	*FIP1-TAP:HIS5*(*Sp)* Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ1*	(Ghaemmaghami et al., 2003)

Table 5: Plasmids used in this study

Plasmid	Description	Reference/Source
pAC 636	*NAB2* CEN *LEU2* AMP	(Green et al., 2002)
pSW3579	*nab2-T178A/S180A* CEN *LEU2* AMP	This study
pSW3580	*nab2-T178E/S180E* CEN *LEU2* AMP	This study
Nab2-pGEX2TK	pGEX2TK GST-*NAB2* AMP	(Lee and Aitchison, 1999)
pSW3610	pGEX2TK GST-*nab2 T178A/S180A* AMP	This study
pSW3465	HIS-*MEX67:MTR2* AMP	This study
pSW1319	GST-Dbp5 AMP	(Alcazar-Roman et al., 2006)

CHAPTER III

FUTURE DIRECTIONS

Introduction

When yeast cells are exposed to heat shock conditions they undergo a dramatic shift in gene expression to quickly and effectively respond to the stress. This includes both retaining non-heat shock mRNA transcripts in the nucleus, and selectively exporting heat shock mRNA transcripts into the cytoplasm where they can be translated. As such, a complete understanding of gene expression during heat shock requires both an understanding of the export of heat shock mRNA, as well as the retention of non-heat shock mRNA. Discussed in this chapter are future experiments aimed at better understanding each of these processes.

It has long been known that *S. cerevisiae* selectively control mRNA export in response to heat shock. However the mechanism for this differential export has remained elusive. Many proteins that are essential for bulk mRNA export are dispensable for heat shock mRNA export, while others are strictly required for both heat shock and non-heat shock mRNA export. Our findings that the mRNA-binding protein Nab2 is phosphorylated and forms intranuclear foci during heat shock are the first reports of Nab2 involvment in the heat shock response. Further, the discovery that Nab2 is phosphorylated by the cell wall integrity pathway MAP kinase Slt2, and that cells defective in this pathway fail to properly retain non-heat shock mRNA is the first report

of MAP kinase signaling affecting mRNA export in *S. cerevisiae*. In this chapter I discuss future avenues of research aimed at answering three distinct questions: 1) What is the mechanism for retention of mRNA during heat shock? 2) How are heat shock mRNAs selectively exported? And, 3) Are other factors known to be involved in bulk mRNA export involved in heat shock mRNA export? Addressing each of these three questions will result in a more detailed understanding not only Nab2 and Slt2 function during heat shock, but of the overall mechanisms of both heat shock mRNA export and non-heat shock mRNA retention.

Investigation into the mechanism of mRNA retention during heat shock

When cells are exposed to heat shock stress, Nab2 is both phosphorylated by the MAP kinase Slt2, and relocalized to punctate intranuclear foci. Strikingly, these two phenotypes are independent of one another. The *slt2Δ* cells lacking Nab2 phosphorylation still form foci, and *mlp1Δ* cells lacking foci formation still exhibiting Nab2 phosphorylation. While the independent occurrence of both Nab2 phosphorylation and foci formation could complicate the understanding of the function of the other, these mutant strains can serve as useful tools to independently dissect the function of each. Genetic screens in yeast are powerful tools that are often used to discover novel factors in cellular processes, exploiting sensitivity to growth temperature in particular yeast strains. Taking advantage of this sensitivity to growth temperature allows for screens aimed at discovering multi-copy suppressors of mutant alleles, as well as genes exhibiting synthetic lethality with a gene of interest. However, these approaches are rendered largely ineffective while studying heat shock, due to cellular death of even wild type

strains after prolonged periods of thermal stress. Due to this, the existence of yeast strains that will allow for dissection of the phenotypes we observe during heat shock, and epitope-tagged strains which can be utilized to isolate mRNPs from heat shocked cells, I propose a largely biochemical approach to understand the mechanism of mRNA retention during heat shock.

My previous work has shown that when Nab2-TAP complexes are purified from cells grown at permissive temperature and cells that have been exposed to heat shock induced stress, reduced levels of the mRNA export receptor Mex67 and nuclear pore complex proteins are associated with Nab2-TAP from heat shocked cells. We speculate that this is due to lack of mRNA export in heat shock induced cells, as Mex67 is essential for export, and NPC proteins that co-purify with Nab2-TAP are presumed to be associated as a result of the Nab-bound mRNPs being actively exported. Consistent with this interpretation, a protein A-tagged version of Yra1, an mRNA export factor that is removed from the mRNP prior to export, is associated with greatly reduced numbers of NPC proteins when compared to protein A-tagged version Mex67 (Oeffinger et al., 2007), or Nab2-TAP, which are both associated with the mRNP throughout the export process. Due to this presumed lack of export of Nab2-bound mRNPs during heat shock, and the lack of retention of mRNA during heat shock in cells lacking *SLT2*, I hypothesize that Slt2 function is responsible for this retention. To confirm this hypothesis, Nab2-TAP purifications can be conducted in wild type cells, and *slt2Δ* cells after heat shock, and complex components can be compared. If this hypothesis is correct, and Slt2 is causing retention, and deletion of *SLT2* is inhibiting this retention, Nab2-TAP should be associated with Mex67 and NPC components in *slt2Δ* cells during heat shock, similar to

non-heat shock conditions, as it will again be associated with actively exporting mRNPs. Further our model predicts that retention is due to the lack of Mex67 recruitment. Consistent with this model, Nab2-TAP is associated with reduced amounts of Mex67 during heat shock, and Mex67 does not localize to Nab2-containing foci during heat shock. If Slt2 function is inhibiting Mex67 recruitment to the mRNP, then deletion of *SLT2* could result in Mex67 now being recruited to Nab2-containing foci. This can be tested directly by examining Mex67-GFP during heat shock induced stress in wild type and *slt2Δ* cells. Mex67 recruitment to Nab2-containing foci in *slt2*Δ would further strengthen this model. However, lack of Mex67 foci formation could potentially be explained in ways that do not conflict with our model. For instance, it is possible that mRNPs retained in foci by Mlp1 are incompletely, or not properly processed, and thus would not have recruited Mex67 in the first place. Consistent with this explanation, Mlp1 and Nab2 have been shown to form foci that contain unspliced mRNA (Galy et al., 2004). As Mex67 recruitment is thought to occur in the late stages of mRNA processing, it is likely that these transcripts were never associated with Mex67. Further, splicing is inhibited during heat shock (Yost and Lindquist, 1991), which, in theory, leads to an increased amount of unspliced mRNA that could be the cause of accumulation with Mlp1 and foci formation. Alternatively, it is possible that if Mex67 is recruited to some number of Mlp1-retained mRNPs that they would thus be rendered export competent, and exported, resulting in no visible Mex67-GFP in foci. These alternate explanations would make a negative result in this experiment difficult to interpret.

To further test this model, Nab2-TAP purifications can also be done in cells lacking *MLP1*, which do not form foci under heat shock conditions. As Nab2 is both

phosphorylated, and forms foci during heat shock, and the two are independent of one another, this serves as an important control to show that retention is specifically due to Slt2, and not Mlp1 foci formation. Consistent with this interpretation, *in situ* hybridization shows that *mlp1Δ* cells still accumulate poly(A) RNA in the nucleus during heat shock. However, because foci do not form the retention pattern is more diffuse, making it difficult to compare quantitatively, and thus some active export of Nab2-bound mRNPS may be occurring.

With regards to Mex67 and NPC protein association with Nab2-TAP in *mlp1Δ* cells during heat shock, there are three broad possibilities, of which any would make important contributions to our model. These are: 1) That Nab2-TAP complexes from *mlp1Δ* cells during heat shock mirror Nab2-TAP complexes from wild type cells during heat shock, with Mex67 reduced ~2 fold, and NPC components reduced drastically, indicating that mRNA retention is not dependent on Mlp1, 2) Nab2-TAP complexes from *mlp1Δ* cells resemble Nab2-TAP complexes from non-heat shocked wild type cells (and what I predict Nab2-TAP complexes from heat shocked *slt2Δ* cells will look like), with similar levels of associated Mex67, and many co-purifying NPC proteins, indicating that Mlp1 can cause retention of Nab2-bound mRNPs independently of Slt2, or 3) something in between these two, where Mex67, and NPC protein association is somewhat reduced versus Nab2-TAP from heat shocked wild type cells, but the reduction in their amounts aren't as severe. While we have shown that bulk mRNA is retained during heat shock in wild type cells, and that this retention is inhibited in *slt2Δ* cells, it is possible that some amount of retained mRNA is exported in cells lacking Mlp1. Consistent with this, unspliced mRNAs, which can form accumulations in foci with Mlp1, are exported to a

greater extent in *mlp1* mutants. If this is the case during heat shock, and some amount of mRNAs which would be retained in wild type cells are being exported, Nab2-TAP samples from *mlp1Δ* cells during heat shock would look like those described in option three above. Examination of the Nab2-TAP complexes in *mlp1Δ* cells will address the question of whether foci formation causes retention of the mRNA, or whether mRNAs that were already destined to be retained bind Mlp1, and thus form foci.

A further set of related experiments comes from analysis of Yra1-TAP, similar to the analysis of Nab2-TAP, described above. Analysis of Nab2-TAP has proved useful in demonstrating that Nab2 is in a physical complex with Mlp1 and Yra1, confirming our co-localization results. It has also proved useful in our initial experiments, and will be used to a similar end in the experiments described above to determine if Nab2 is associated with actively exporting mRNPs. Unlike Nab2, Yra1 is removed from the mRNP before export (Strasser and Hurt, 2000) , and thus protein complexes co-purifying with Yra1 are very distinct from those that co-purify with Nab2 (Oeffinger et al., 2007), despite the fact that they are presumed to be on the same set of transcripts (Iglesias et al.). The differences are two fold, with Yra1 complexes, 1) having greatly decreased amounts of NPC proteins associated with them, and 2) having a broader array of mRNA-binding proteins associated with them. Both of these can be attributed, at least in part, to Yra1 removal before export. NPC components are thought to co-purify with most mRNA-binding proteins due to their associated mRNPs being actively exported. Since Yra1 is removed prior to export, NPC components are greatly reduced. Also, Yra1 is not the only protein to be removed prior to export, thus Yra1-associated protein complexes have more mRNA-binding proteins associated with them then Nab2 complexes, as Nab2

complexes are composed largely of actively exporting mRNPs that have had many of their mRNA binding proteins already removed. While the association of Nab2-TAP complexes with NPC proteins has been, and will be, utilized in the above experiments, the association of Yra1 with a plethora of mRNA-biding proteins will be utilized in the experiments below.

The first level of this analysis is on Yra1-TAP from wild type cells, before or after heat shock. As Nab2, Mlp1, and Mex67 have all been demonstrated to co-purify with Yra1 complexes (Oeffinger et al., 2007), and Yra1, Nab2, and Mlp1, but not Mex67 form foci during heat shock, I anticipate that these protein's association with Yra1-TAP will mirror Nab2-TAP. That is that Mlp1, and Nab2 will exhibit increased association with Yra1-TAP during heat shock, and Mex67 association will be decreased. These altered interactions would serve as a useful control, ensuring that Yra1-TAP is co-purifying with the same foci-associated mRNPs as Nab2-TAP. Currently we know that Nab2, and Yra1 form foci with, and dependent on Mlp1. While we have not yet shown definitively that there is RNA in the foci, I believe that there is, based on a number of observations. First, poly(A) accumulation is granular, forming intranuclear foci similar to the Mlp1/Yra1/Nab2 foci during heat shock, and this granular pattern is no longer seen in *mlp1Δ* cells, which lack foci. Second, Mlp1, and Nab2 have been demonstrated to form foci bound to unspliced mRNA (Galy et al., 2004). And lastly, Nab2-TAP purifications show increased association of Pab1, Tho2, and Stm1 during heat shock, all of which are mRNA-binding proteins that are not known to directly interact with Nab2. However, while the evidence is strong that there is mRNA in the Nab2 foci, we will test this directly, using co-localization of Nab2 and poly(A) RNA. Demonstrating that there is

mRNA in the foci, and that Nab2, and Mlp1 are enriched on Yra1-TAP during heat shock provides confidence that we are isolating mRNPs from foci, and that we can survey the rest of the Yra1-TAP co-purifying proteins that are enriched during heat shock to further probe the function of Mlp1/Nab2/Yra1 foci. For instance, if the transcripts retained in foci are not completely processed, there could be an increase in mRNA maturation factors, such as splicing factors, or 3'-end processing factors enriched on Yra1-TAP during heat shock that would be indicative of this. If this is the case, and splicing factors are indeed preferentially isolated with Yra1-TAP during heat shock, a more direct test can be performed to show that there is, in fact, unspliced mRNA in the foci. This can be tested by transforming cells with galactose inducible reporter constructs that either have, or lack, an intron. By adding galactose to the media, the construct is expressed, and cellular localization can be assayed by *in situ* hybridization using a Cy5 labeled probe, as in Galy et. al. 2004. By co-staining cells for Mlp1-GFP, and the reporter constructs, it can be determined if intron-containing transcripts are preferentially retained within the foci.

Along with possible insights into the mRNA maturation state of retained foci, Yra1-TAP could also provide insight into the mRNA-binding protein differences between heat shock, and non-heat shock transcripts. Yra1 is likely bound exclusively to non-heat shock mRNA, as it is retained in foci, and not necessary for heat shock mRNA export, and as such, the protein profile of Yra1-TAP during heat shock could represent a set of mRNA-binding proteins that are enriched on retained non-heat shock transcripts. In order to begin to draw such a conclusion, RT PCR would need to be performed on Yra1-TAP samples from heat shocked cells to ensure that no heat shock transcripts are present.

If indeed this is the case, co-purifying proteins, especially those enriched during heat shock, can be analyzed individually for potential roles in retention. Each can be put through a battery of assays, similar to those used to characterize Nab2 during heat shock. (The *S. cerevisiae* genome wide collections have ~4,000 TAP-tagged, and GFP-tagged strains each in them (~2/3 of the *S. cerevisiae* genome), and will serve as invaluable tools for many of the experiments discussed below, and later in this chapter.) These include assaying localization during heat shock. If a protein is indeed bound to the mRNA retained in foci, a GFP-tagged version will likely show association with the foci during heat shock. Alterations in post translational modifications can also be tested, by SDS-PAGE, and immunoblotting.

The mechanism for mRNA retention in foci is unknown, and post translational modification of an mRNA-binding protein or proteins could be an intriguing way for this to happen. If a possible post translational modification is detected during heat shock, this can be tested for phosphorylation directly. If phosphorylated residues are identified, mutant strains harboring non-phosphorylatable or phospho-mimetic mutants will be constructed. These mutant strains can then be tested to see if they affect retention of mRNA, or foci formation during heat shock. They will also be tested for defects in heat shock mRNA export. In theory, they are not likely to have heat shock mRNA export defects, as they are retained in foci. However, this is an important control to demonstrate that modification is potentially affecting retention and not heat shock mRNA export. If, on the other hand, mutants do have an export defect, they can be included in the list of proteins necessary for heat shock mRNA export, and can be further characterized in that role. A further control, to ensure that enriched co-purifying proteins on Yra1 during heat

shock are due to foci-association is to compare Yra1-TAP from heat shock in wild-type cells to Yra1-TAP from heat shocked cells in an *mlp1Δ* strain. Since foci do not form in *mlp1Δ* enriched proteins during heat shock in this strain would be independent of foci formation.

One further proteomics based experiment could be done to address the difference in retention phenotype of *slt2Δ* and *nab2-T178A/S180A*, the non-phosphorylatable version of Nab2. Our data show that deletion of *SLT2* results in lack of retention of mRNA, but that lack of its phosphorylation of Nab2 is not sufficient for this effect. This indicates that there is likely something different between the retained mRNPs in *nab2-T178A/S180A*, and the non-retained mRNPs in *slt2Δ*.

Utilizing the fact that Yra1-TAP is able to purify mRNA with a multitude of mRNA-binding proteins, we can determine complex components from Yra1-TAP samples from heat shocked cells in *slt2Δ* and *nab2-T178A/S180A*, and compare the protein profiles. If there is indeed a difference in the mRNP composition that is causing the different phenotypes in these strains, this could determine what it is. It is possible that this could be a either a direct Slt2 substrate (a discussion of a search for Slt2 substrates is later in this chapter), or a protein that fails to get recruited to the mRNP due to an upstream effect of phosphorylation of another Slt2 substrate that is not bound to the exporting mRNP. Further, it is possible that a protein causing retention either be associated with the mRNP in *slt2Δ*, and not *nab2-T178A/S180A*, or vice versa. This could be due to Slt2 function either 1) causing a protein to stay bound, when its removal is necessary for export, or 2) Slt2 function causing removal of a protein whose association is necessary for export. This experiment could detect a protein in either case,

and thus any differential binding proteins between the two strains would be examined closely. A possible caveat to this is that mRNPs are presumably being exported in *slt2Δ*, and thus differences in *slt2Δ*, and *nab2-T178A/S180A* could be due to cytoplasmic alterations of the mRNP that are not reflective of the nuclear changes that alter retention. To control for this, these isolations could be done in double mutant strains, harboring a mutant that acts late in the mRNA export pathway, alongside either the *slt2Δ* or *nab2-T178A/S180A* mutations. A mutant in the mRNA export factor, *GLE1*, could be potentially useful to this end, as Gle1 is believed to act specifically at the late stages of mRNA exoprt. If this approach proves successful in detecting an mRNA-binding protein that is differentially associated with Yra1-TAP in *slt2Δ*, and *nab2-T178A/S180A* during heat shock, many follow up experiments can be done to test its role in retention, including testing for predicted MAP kinase phosphorylation sites, testing for altered migration on SDS-PAGE by immunoblotting, and testing for re-localization during heat shock. If this candidate protein does, in fact, contain predicted MAP kinase phosphorylation sites, or exhibits gel shift by SDS-PAGE during heat shock, testing for phosphorylation sites can be performed as described above. If it is phosphorylated, and sites are mapped, phospho-dead mutants can be constructed in both wild type cells, and *nab2-T178A/S180A* cells, and these will be tested for lack of retention of mRNA during heat shock. If cells harboring this mutant alone show lack of retention, it could be considered as the target of Slt2 responsible for retention. To validate that, phoshomimetic mutants will be made, and tested to see if a phosphorylated version of this protein is sufficient to cause retention in non-heat shocked cells. Alternatively, it is possible that a phospho-dead mutant of this protein will act like *nab2-T178A/S180A*, and

not recapitulate the *slt2Δ* phenotype. In this case, it would be tested for lack of retention in combination with *nab2-T178A/S180A*, as perhaps phosphorylation of both proteins is necessary for retention. Again, if this is the case the phosphomimetic would be tested in combination with *nab2-T178E/S180E* to see if combined the two phosphomimetics would prove sufficient to elicit retention in non-heat shocked cells.

If a protein is found on Yra1-TAP in *slt2Δ*, and not *nab2-T178A/S180A*, but it is not phosphorylated, any existing mutants or a null mutant, if these exist, could be tested in the same set of experiments as a phospho-protein above. If this were the case, it is possible that despite not being phosphorylated, the identified protein is not associated with the mRNP due to Slt2 phosphorylation of an upstream component that blocks its recruitment, and as such a deletion mutant, or a mutant that does not associate with mRNPs could potentially cause retention alone, or in combination with *nab2-T178A/S180A*, as described above.

Taken together, this set of proteomics experiments, and the follow up experiments that they result in, can provide a more in depth knowledge of how non-heat shock mRNA are retained in the nucleus during heat shock, addressing, among other things, the role of Mlp1 in retention, the function of Mlp1/Nab2/Yra1 foci, the composition of nuclear retained-mRNPs, and the potential differential mRNP composition that results in retention of mRNA in *slt2Δ*, but not *nab2-T178A/S180A*.

Identification of additional Slt2 substrates

Our identification of Nab2 as an Slt2 phosphorylation substrate is the first link between MAP kinase signaling and mRNA export or retention in *S. cerevisiae*, with cells

lacking *SLT2* failing to accumulate non-heat shock mRNA in the nucleus under heat shock conditions. Due to Nab2 being the only Slt2 target known to function in mRNA export, and the observation that Nab2 itself accumulates in the nucleus during heat shock, we propose that Nab2 phosphorylation is important for this phenotype. However cells harboring only a non-phosphorylatable version of *nab2* do not exhibit the same phenotype as cells lacking *SLT2*, the Nab2 kinase. A likely explanation is that there is another, as of yet undefined Slt2 substrate, phosphorylation of which is also necessary for mRNA retention. Therefore, to fully understand the role of Slt2 on mRNA retention, other Slt2 targets must be identified and characterized.

A candidate approach was employed to test proteins that were annotated as being nuclear and RNA-binding with predicted MAP kinase sites. However, this proved unsuccessful. There are multiple reasons for why this could have been the case, including the target potentially not being an RNA-binding protein, not being annotated as nuclear or RNA-binding, not having predicted MAP kinase sites, or not exhibiting a noticeable shift in migration by SDS-PAGE when phosphorylated. Thus an unbiased approach at identifying Slt2 targets should be performed. In fact a recent study addressed a similar question identifying ERK kinase substrates utilizing a tissue culture cell line in which the Raf-MEK-ERK pathway could be either rapidly induced or inhibited by a drug addition (Kosako et al., 2009). A similar approach could be used in *S. cerevisiae* to discover novel Slt2 substrates. This would entail growing both wild type and *slt2Δ* cells under heat shock conditions, and purifying phosphorylated proteins on an immobilized metal ion-affinity chromatography (IMAC) column. Proteins eluted from the wild type samples could then be labeled with a Cy3 dye, and proteins from the *slt2Δ* cells labeled

with Cy5 dye, and the samples could be mixed and separated on the same two dimensional (2D) gel. Using this workflow would allow for identification by mass spectrometry of proteins specifically phosphorylated by, or downstream of, Slt2, as will be detected by increased signal from the wild type sample versus the *slt2Δ* sample. Once identified by this method, phosphorylation sites could be confirmed both *in vivo* and *in vitro*. The phosphorylation sites of Slt2 targets can then be mapped by mass spectrometry, or predicted by phosphorylation prediction programs, and mutated to act as phosphodead or phosphomimetic mutants. Phosphodead mutant strains can then be tested for poly(A^+) accumulation during heat shock to determine if they can recapitulate the *slt2Δ* phenotype, and phosphomimetic strains tested to determine if they are sufficient to induce retention of mRNA without heat shock. If these are not the case, strains containing higher order combinations of phosphodead, and phosphomimetic mutants will be constructed, including *nab2* mutants, and mutants identified from this assay. Strains containing multiple phosphodead versions of Slt2 targets will be assayed to determine if lack of phosphorylation of two or more specific Slt2 targets is sufficient to inhibit retention. Conversely, strains containing multiple phosphomimetic versions of Slt2 targets will be tested to determine if they are sufficient to promote mRNA retention in non-heat shocked cells. Discovery of a target, or combinations of targets, that can recapitulate the *slt2Δ* phenotype during heat shock, and potentially cause retention of mRNA without heat shock, will result in specific hypotheses for the mechanism of retention. For instance, if mutation of another mRNA binding protein, in combination with *nab2* is sufficient to recapitulate the *slt2Δ* phenotype, it may be hypothesized that phosphorylation of both of these factors is necessary for lack of Mex67:Mtr2 recruitment.

Another possible explanation is that an upstream pre-mRNA processing factor is essential for retention, which could indicate that non-heat shock mRNA are not being properly processed, and are thus retained during heat shock. Once a protein, or combination of proteins is found that can recapitulate the *slt2Δ* phenotype, these hypotheses can be tested directly.

Investigation into the mechanism of heat shock mRNA export

To date nuclear export of all mRNA species tested in *S. cerevisiae* require the nuclear export receptor heterodimer Mex67:Mtr2. This includes both heat shock, and non-heat shock transcripts. Additionally, Mex67 alone binds RNA weakly and current models of mRNA export assume that all transcripts use an adaptor protein, or proteins, to recruit Mex67:Mtr2 to the mRNP. To date three such adaptor proteins have been proposed, Yra1, Npl3, and Nab2 (Gilbert and Guthrie, 2004; Strasser and Hurt, 2000). Strikingly, while each of these proteins is essential for both cell viability, and bulk mRNA export, each is dispensable for heat shock mRNA export (Krebber et al., 1999; Rollenhagen et al., 2007). Taken together, these results raise the question: How is Mex67:Mtr2 recruited to heat shock mRNA? Two possible answers to this question, and experiments to test each of them will be addressed in this section. These possibilities are: 1) That there is another, as of yet unidentified, adaptor protein for Mex67 that is used by heat shock mRNA, and 2) That heat shock transcripts use two or more adaptor proteins, potentially either a combination of previously described adaptors, a combination of previously uncharacterized receptors, or a combination of both, but that there is

redundancy and lack of no one individual adaptor is sufficient to cause a heat shock mRNA export defect.

In order to identify a possible uncharacterized adaptor protein responsible for Mex67:Mtr2 recruitment to the mRNP, a biochemical approach will be employed. If indeed Mex67:Mtr2 is being recruited to heat shock protein transcripts by an uncharacterized adaptor, it could be identified by association with either Mex67, or heat shock transcripts. To address this question, two related approaches, could be utilized. The first is to isolate Mex67-TAP from both heat shocked, and non-heat shocked cells, and identify co-purifrying proteins by mass spectrometry. In support of this approach, two of the three previous characterized Mex67 adaptors, Npl3, and Yra1, have been found associated with Mex67-PrA isolations (Oeffinger et al., 2007). However, Mex67 has never been purified from cells under heat shock conditions. If a novel adaptor is being used, it could be enriched with Mex67 specifically during heat shock. Alternatively, a novel adaptor could also associate with non-heat shock mRNA, and thus not be enriched with Mex67-TAP during heat shock. As such, identification of mRNA-binding proteins, or potential mRNA-binding proteins associated with Mex67-TAP could be tested as possible Mex67 adaptors, with specific interest on any such protein or proteins that are enriched during heat shock. To test this, strains lacking these proteins (if deletion strains are viable), or strains harboring known mutations in the genes encoding these proteins (if they exist), could be tested for heat shock mRNA export defects. If this protein is the Mex67:Mtr2 adaptor during heat shock, a heat shock mRNA export defect is likely to be detected in a strain lacking, or harboring a defective copy of, this protein. Further, if this protein is to be considered a *bona fide* adaptor, it must bind Mex67

directly to recruit it to the mRNP. This could be tested directly *in vitro* using recombinant purified proteins. This can also be supported *in vivo*, by treating Mex67-TAP samples with RNase before isolation, demonstrating that the interaction is not bridged by RNA. An identified protein that directly binds Mex67, but is not sufficient to elicit a heat shock mRNA export, could still function as a potential adaptor if there are other adaptors that act in a redundant fashion. This will be discussed in further depth later in this chapter.

A second way to address this is to specifically isolate the mRNP from a single heat shock gene, and compare it to the mRNP from a non-heat shock gene. This can be accomplished utilizing the MS2 binding system. The bacteriophage MS2 coat protein binds a specific stem-loop structure in the viral RNA, allowing encapsidation of the viral genome (Peabody, 1993). As such, constructing MS2 stem-loops into a gene of interest, and co-expressing an epitope-tagged version of the MS2-binding protein allows for analysis of a single mRNA species *in vivo*. This system can be applied to the case of heat shock mRNA export by designing two reporter plasmids, one with MS2 stem-loops in a heat shock gene, and one with MS2 stem loops in a non-heat shock gene. Each can then be purified with MS2-TAP from heat shocked cells, and co-purifying proteins can be analyzed by mass spectrometry. Mex67-Mtr2 adaptors for heat shock transcripts will likely be enriched on the heat shock mRNA versus the non-heat shock mRNA. This approach could prove more straightforward than utilizing Mex67-TAP, as Mex67 will bind to multiple transcripts, while the MS2 system is designed to purify only a single mRNA species, which in theory will result in a more homogenous purification. As with the Mex67-TAP experiment, candidates for potential adaptors can be tested individually

for Mex67-binding ability, and heat shock mRNA export defects. As with the *slt2Δ/nab2-T178A/S180A* comparison, a potential caveat to this experiment is that the heat shock transcript will be getting exported into the cytoplasm, while the non-heat shock transcript will be retained in the nucleus, which could lead to cytoplasmic removal of the to be identified adaptor. Again, an mRNA export mutant, such as *gle1*, can be used to block export to overcome this potential problem.

A second scenario for how Mex67 could be getting recruited to heat shock mRNA is through a combination of the previously characterized adaptors, or perhaps a combination of previously characterized, and as of yet uncharacterized receptors. Mutation of the genes encoding any of the three characterized receptors results in a bulk poly(A^+) export defect, but not a heat shock mRNA export defect. While this means that lack of any one of the known Mex67:Mtr2 receptors alone is not sufficient to cause a heat shock mRNA export defect, it remains possible that these receptors are being used by heat shock protein transcripts, but that each transcript is using multiple adaptors, and loss of only one is not sufficient to block export. It is intriguing to think that this might be a way in which heat shock transcripts help to ensure that they are preferentially exported, as recruiting many receptors could be a way to ensure that they are rapidly exported, and that non-heat shock transcripts are not. As there are only three known receptors and mutant strains defective in each exist, strains harboring mutations in either two or all three of the known adaptors could be tested for heat shock mRNA export defects. It would be particularly interesting to test combinations of these proteins that are found associated with the MS2 heat shock mRNA above. Further, proteins identified as associated with the MS2 heat shock transcript above, that also bind Mex67, but do not

result in heat shock mRNA export defects when their genes are mutated, can be included in these combinatorial pairs to see if they, along with a known receptor, are responsible for Mex67 recruitment to heat shock transcripts.

Identification of other mRNA export factors that play a role in heat shock mRNA export, or non-heat shock mRNA retention

While many proteins that are known to play a role in bulk mRNA export have also been tested for a role in heat shock mRNA export, there are many known mRNA export factors whose potential role in heat shock mRNA export has not yet been investigated. Further, even fewer of these factors have been tested for roles in mRNA retention during heat shock. Generally, studies in the field have focused on one factor at a time, and tested only for heat shock mRNA export. In order to gain further insight into the process, a more global analysis of both heat shock mRNA export and retention of non-heat shock mRNA could be performed using existing mutant strains known to be involved in bulk mRNA export. Factors that have been shown to play a role in bulk mRNA export that have not been tested for a role in heat shock mRNA export, or non-heat shock mRNA retention, include the following:

- The mRNA binding proteins Npl3, Gbp2, Hrb1, and Tho1. Interestingly, Gbp2, and Hrb1 have sequence similarities to Npl3, one of the previously described Mex67:Mtr2 adaptor proteins. While *npl3* mutants have been tested for, and do not have heat shock mRNA export defects, tests for mRNA retention have not been conducted. Mutants in *gbp2*, *hrb1*, *tho1* have been tested for neither heat

shock mRNA export defects, nor non-heat shock mRNA retention. Strains exist to test all four of these proteins, including viable null strains for *gbp2*, *hrb1*, and *tho1* and previously characterized mutant strains for *npl3*.

- Transcription export complex (TREX) complex members Tho2, Hpr1, Mft1, Thp2, Sub2, and Yra1. TREX is the first described protein complex characterized as affecting both transcription, and mRNA export. Mutant strains in each of the complex members have been tested for heat shock mRNA export, but none have been tested for a role in non-heat shock mRNA retention. Testing these mutants for defects in mRNA retention could prove interesting based on the observation that Yra1 is retained in foci during heat shock. Viable null mutants exist in *tho2*, *hpr1*, *mft1*, and *thp2*, and published mutants exist in *yra1*, and *sub2*, which can be tested.
- Transcription export complex 2 (TREX-2) complex members Sac3, Sus1, and Thp1. TREX-2 is the second described protein complex characterized as affecting both transcription, and mRNA export. Complex mutants have been tested for neither heat shock mRNA export, nor non-heat shock mRNA retention. The mechanism by which TREX-2 acts in mRNA export is currently not clear. Viable null strains exist to test each of these three proteins.

In order to make these tests comprehensive, each of the above mutants could be tested for both heat shock mRNA export defects, and for retention of mRNA during heat shock, as discussed above. Proteins found to play a role in either retention, or heat shock mRNA export can be further characterized, including analyses of localization, and potential heat shock-induced posttranslational modifications. A large scale analysis such as this could

prove vital to understanding the mechanisms of both heat shock export, and non-heat shock retention. Interestingly, many mRNA-binding proteins, and thus potential Mex67:Mtr2 adaptors are included in the above list of proteins. If one of these proteins is acting as the main Mex67:Mtr2 adaptor during heat shock, these analyses could allow for its identification, as mutants lacking this protein could display heat shock mRNA export defects. Of further note, while many of the proteins proposed to be examined are non-essential, and thus not vital for bulk mRNA export, this does not mean that they are not vital for heat shock mRNA export. In line with this, Nup42, a protein that is neither essential for viability under normal growth conditions nor bulk mRNA export, is essential for heat shock mRNA export. Understanding mRNA export is particularly difficult due to the connections to upstream, and downstream processes, as well as due to the large number of factors that are involved. An analysis such as this will allow for more pointed experiments on proteins found to play a role in heat shock mRNA export or non-heat shock mRNA retention. This will also allow for inclusion or exclusion of the factors known to be involved in non-heat shock mRNA export into an ever evolving model of heat shock mRNA export.

Concluding Remarks

While the studies described in this chapter are focused specifically on understanding the mechanisms by which mRNA export, and mRNA retention are controlled during heat shock in *S. cerevisiae*, they have the potential to impact a much broader range of fields. Study of heat shock mRNA export is of interest not only because it is a part of an evolutionarily conserved stress response pathway, but also because it

highlights the fact that export of all mRNA species is not carried out in the same manner. *S. cerevisiae* serves as a wonderful model system to address heat shock export, as it is amenable to genetic alterations, as well as biochemical and cell biological techniques. However, future studies of mRNA export in response to stress will expand into differing stress conditions, and more complex systems. This could result in heat shock mRNA export in yeast becoming a paradigm for how the cell can specifically alter its expression pattern in response to stress by controlling what mRNA reaches the translation machinery in the cytoplasm. For instance, mRNA export in response to stress in *S. cerevisiae* is not the same for heat shock stress as it is for high osmotic stress. Each elicits a stress response, and affects mRNA export, but in different ways.

Our findings that MAP kinase signaling plays a role in the mRNA export pathway during heat shock makes it intriguing to think that multiple cellular signaling pathways can differentially affect mRNA export in response to different stress conditions. Further, these studies lay the groundwork for understanding mRNA export in response to stress in higher eukaryotes. Due to its utility as a model system, many mRNA export studies are carried out in *S. cerevisiae*. However, many of the mRNA export factors discovered in yeast are evolutionarily conserved, and involved in mRNA export throughout eukaryotes, including humans cells. Thus, a detailed description of heat shock mRNA export in yeast could lead to models to test mRNA export in response to cellular stress in human cells. The heat shock response is conserved from yeast to humans, and as such a mechanism for how it is carried out in yeast could prove instrumental in understanding it is carried out in human cells. Further, this could lead to potential mechanisms for how human cells control mRNA export during a variety of other cellular stresses, including oxidative

stress, and UV stress. In conclusion, understanding the mechanisms underlying mRNA export is not only mandatory for understanding the basic gene expression pathway, but also for understanding how cells cope with, and survive through stress conditions.

Appendix

A. PURIFICATION OF NAB2-PROTEIN A COMPLEXES FROM mRNA EXPORT MUTANTS

The lifecycle of an mRNA in complex, with multiple proteins associating and dissociating with the mRNP throughout its existence, affecting its maturation at every step. Each process that the mRNA undergoes, from nuclear capping, splicing, and 3' end formation, to cytoplasmic translation and degradation, are the result of the particular set of mRNA-binding proteins associated with the transcript at that time. As such, understanding how mRNP dynamics occurs is fundamental to understanding gene expression. In these studies, we set out to understand how mRNP composition changes during nuclear mRNA export. Broadly, mRNA export can be broken into three distinct phases. First, a fully processed, mature mRNP must recruit an export receptor (Mex67 in *Saccharomyces cerevisiae*), which docks it to the nuclear pore complex (NPC), so that it can be exported. Second, through the bridged interaction of the export receptor, and the NPC, the mRNP is extruded through the pore, and passed through the pore to the cytoplasmic side. Lastly, the mRNP is remodeled by a Gle1/Dbp5/IP_6 complex on the cytoplasmic face of the NPC, allowing for release into the cytoplasm. Evidence from studies of the Balbiani ring mRNP from the salivary cells of *Chironomus tentans* (Daneholt 2001), indicates that proteins both associate, and dissociate with the mRNP throughout this process. In order to understand the precise molecular changes that occur during this process, we designed an experimental setup in *S. cerevisiae* whereby mRNPs

blocked at distinct steps in the mRNA export pathway could be purified, and proteins components analyzed. To this end, strains harboring a protein A (PrA) tagged version of the mRNA-binding protein Nab2 were constructed in multiple temperature sensitive mutant backgrounds, defective at different points in the mRNA export pathway. Nab2 was chosen to isolate mRNPs for a number of reasons, including it is abundant, essential for mRNA export, and shuttles from the nucleus to the cytoplasm bound to the mRNP, all of which, in theory, will make it a useful tool to isolate mRNPs at each step in the export process. Three mutant strains were chosen to perform Nab2-PrA isolations from. These were *mex67-5*, *gle1-4*, and *nup116Δ*. These strains were chosen because they are hypothesized to block mRNA export at three distinct steps (Figure A1). Mutation in *MEX67* will result in a defective export receptor, blocking mRNA export at an early step, as mRNPs will not be able to dock to the NPC (Segref et. al, 1997). Mutation in *GLE1* results in a defective release mechanism from the NPC, blocking mRNA export at an intermediate step (Murphy and Wente, 1996). Deletion of the nucleoporin, *NUP116*, causes a defect in NPC and nuclear envelope architecture, whereby the outer nuclear envelope forms herniations over the NPCs, trapping would be exporting cargo (Wente and Blobel, 1993). This is believed to block mRNA export at the latest possible step, as mRNPs will be fully processed, but physically blocked from entrance in the cytoplasm. By isolating Nab2-PrA from temperature arrested cells in each of these mutant backgrounds, it may be able to discover the precise biochemical changes that occur on an mRNP as it is being exported from the nucleus to the cytoplasm. In order to ensure that the isolated mRNPs were reflective of the native mRNPs, a cryogenic harvesting and lysis protocol was employed, followed by rapid purification on IgG-coated magnetic

beads (Oeffinger et al). The following figures detail this work, including purification of Nab2-PrA each of the mutants discussed above, at both permissive temperature, and non-permissive temperature. Nab2-PrA was also isolated from wild type cells grown at 23ºC or after shift to 37ºC for one hour. Proteomic analysis of these wild type strains is included within. Lastly, temperature shifted samples of Nab2-PrA purifications from *mex67-5* and *gle1-4* are compared using differential gel electrophoresis (DIGE). This experiment shows that Mex67 and Mtr2 are no longer associated with Nab2-PrA mRNPs at non-permissive temperature in *mex67-5* cells.

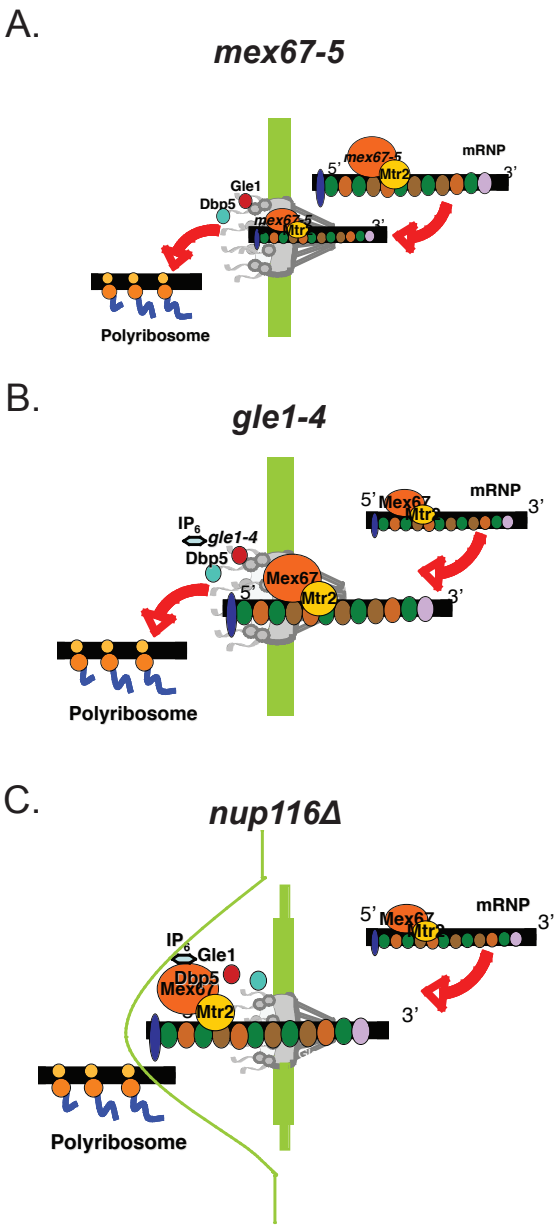


Figure A1. Mutants strains in *S. cerevisiae* theoretically block mRNA export at distinct steps at non-permissive temperature. (A) *mex67-5* is an export in the mRNA export receptor block is proposed to block mRNA export at an early step, as the mRNP will not be able to associate with the nuclear pore complex (NPC). (B) *gle1-4* is proposed to block mRNA export at an intermediate step. mRNPs in *gle1-4* have a functional export receptor, but can not be released from the NPC, due to the mutation in *GLE1*. (C) *nup116*Δ cells are proposed to block mRNA export at a late step. Cells lacking *NUP116* form herniations in the nuclear envelope, covering the NPC, and physically blocking nucleo-cytoplamic trafficking. We hypothesize that blocked mRNPs are fully modified, and ready for cytoplasmic release, but cannot reach the cytoplasm due to these herniations.

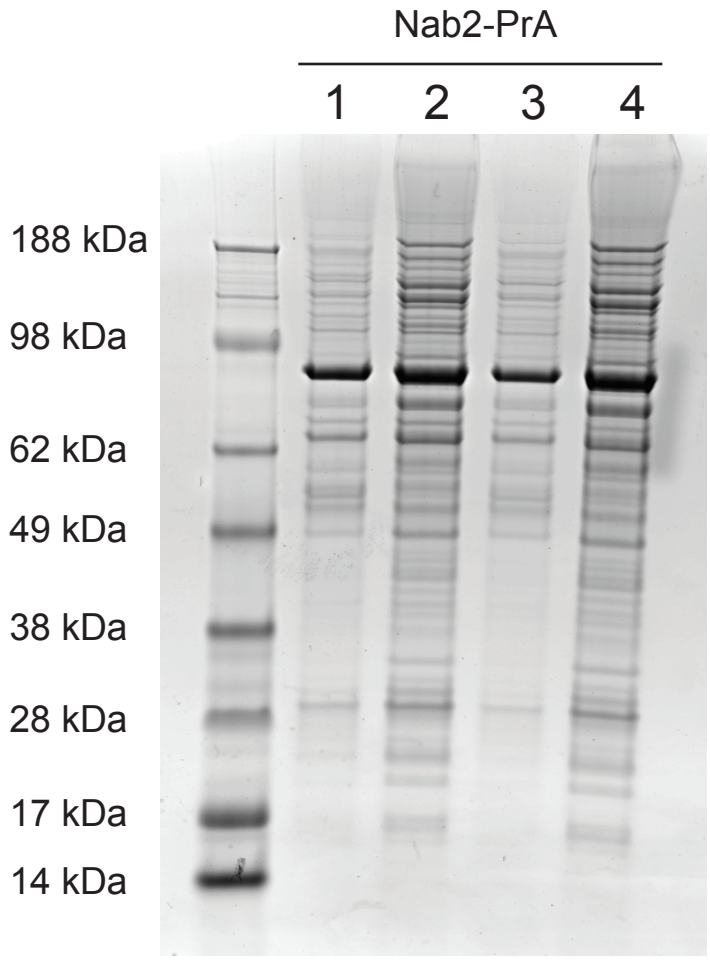


Figure A2. Nab2-PrA yields are highest in buffer containing magnesium. Nab2-PrA complexes were purified under different conditions from wild type cells grown at 23°C in YPD. Purifications represented in lanes 1 and 3 were performed in extraction buffer lacking $MgCl_2$ (20 mM HEPES pH=7.4, 110 mM potassium acetate, 100 mM NaCl .5% Triton X-100, .1% Tween 20, 1mM PMSF, 4 micrograms/ml pepstatin A, 1:5000 Antifoam emulsion B) whereas purifications in lanes 2 and 4 contained 2mM $MgCl_2$ in the extraction buffer. All purifications were bound to beads for 30 minutes at 4°C. Lanes 3 and 4 were treated with DNase A during purifications.

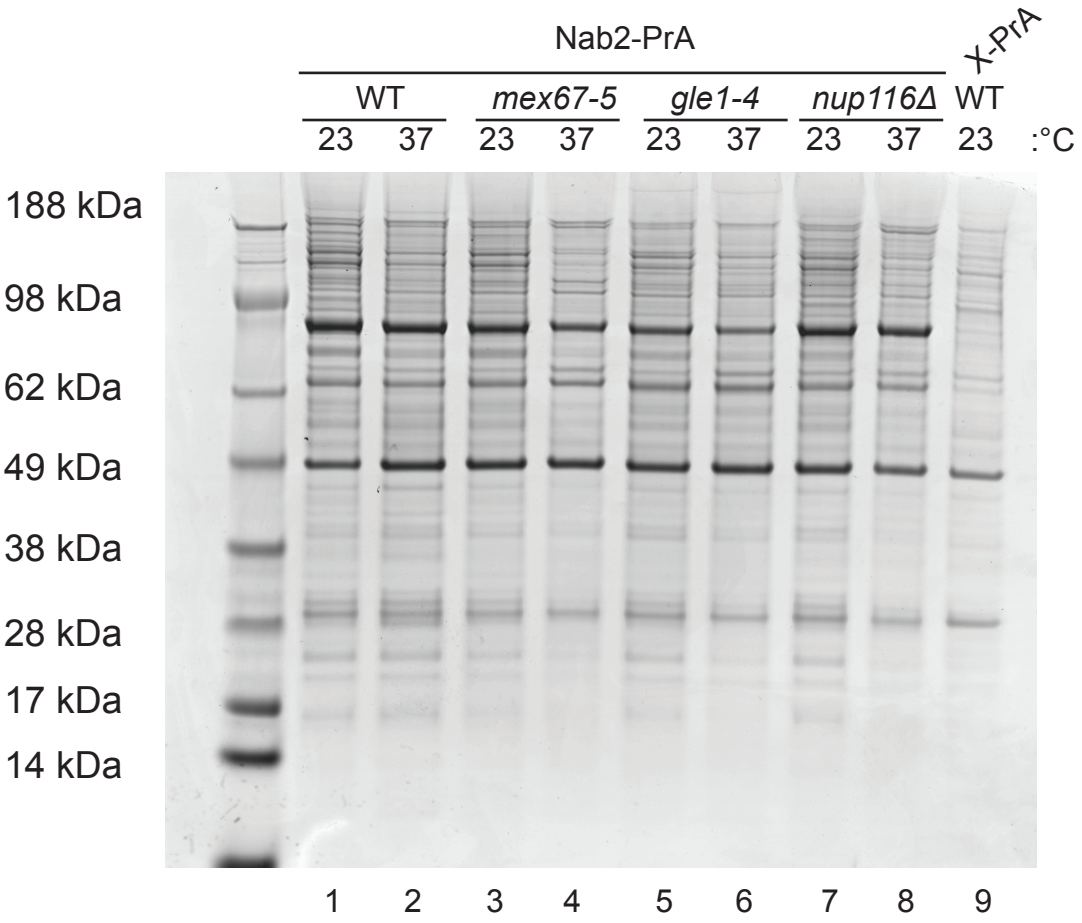


Figure A3. Nab2-PrA complex analysis in wild type, *mex67-5*, *gle1-4*, and *nup116*Δ, from permissive, and non-permissive temperature under low stringency binding conditions. Strains were grown in YPD at 23°C until late log phase (OD_{600} ~ .8), and one half of culture was harvested. Equal amounts of 65°C YPD were added to remaining culture, and grown at 37°C for 1 hour before harvesting to induce the phenotype of the mRNA export mutants. In order to test the sensitivity of complex purification of Nab2-PrA, purifications were carried out in TBT buffer (20 mM HEPES pH=7.4, 110 mM potassium acetate, 2 mM $MgCl_2$, .5% Triton X-100, .1% Tween 20, 1mM PMSF, 4 micrograms/ml pepstatin A, 1:5000 Antifoam emulsion B) without addition of NaCl for 30 minutes at 4°C. Each lane represents 1 gram of cell powder. A purification from a strain harboring PrA not fused to an endogenous protein was included as a negative control (lane 9).

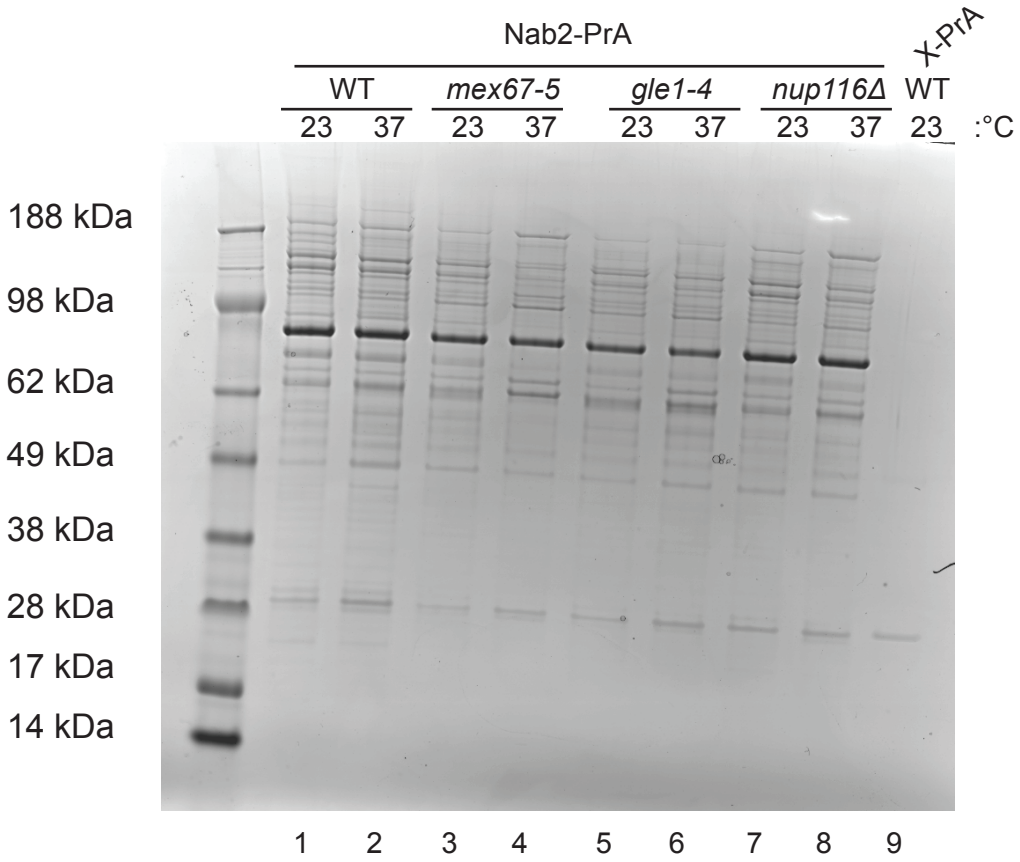


Figure A4. Nab2-PrA complex analysis in wild type, *mex67-5*, *gle1-4*, and *nup116*Δ, from permissive, and non-permissive temperature under medium stringency binding conditions. Strains were grown in YPD at 23°C until late log phase (OD_{600} ~ .8), and one half of culture was harvested. Equal amounts of 65°C YPD were added to remaining culture, and grown at 37°C for 1 hour before harvesting to induce the phenotype of the mRNA export mutants. In order to test the sensitivity of complex purification of Nab2-PrA, purifications were carried out in TBT buffer (20 mM HEPES pH=7.4, 110 mM potassium acetate, 2 mM $MgCl_2$, .5% Triton X-100, .1% Tween 20, 1mM PMSF, 4µg/ml pepstatin A, 1:5000 Antifoam emulsion B) + 100mM NaCl for 30 minutes at 4°C.. Each lane represents 1 gram of cell powder. A purification from a strain harboring PrA not fused to an endogenous protein was included as a negative control (lane 9).

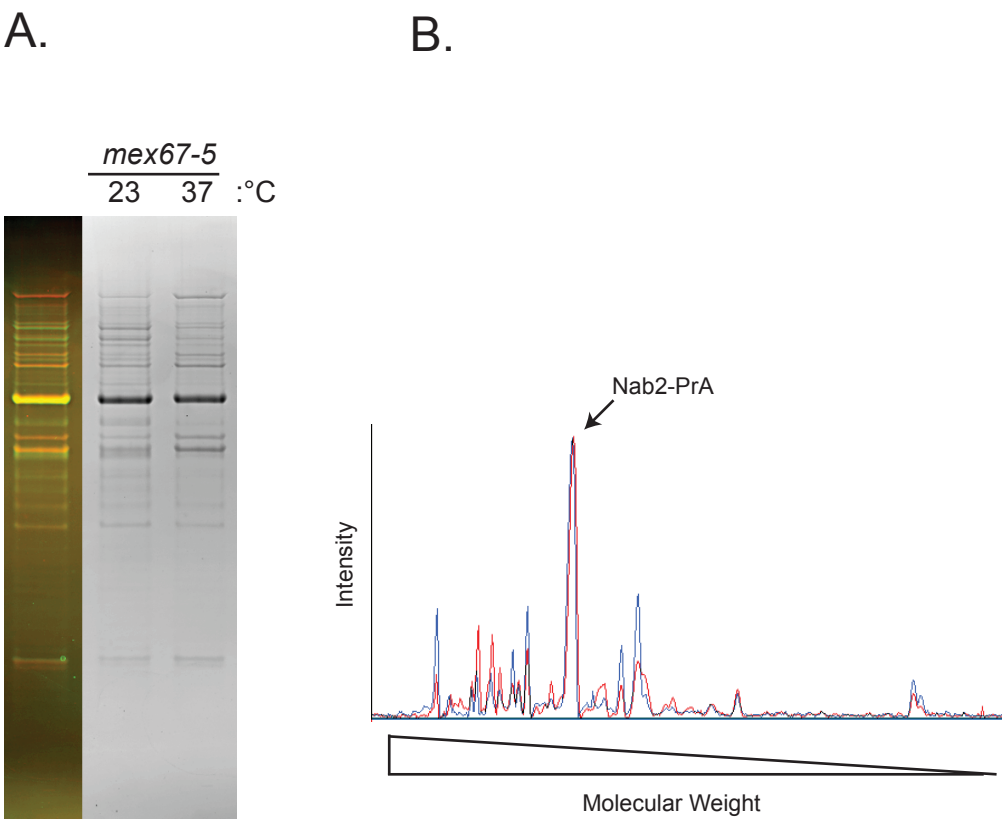


Figure A5. Analysis of Nab2-PrA from *mex67-5* at 23°C vs. 37°C. (A) Nab2-PrA samples from *mex67-5* in Figure 5 were pseudocolored, and merged using ImageJ. The 23°C sample was colored green, and the 37°C sample, red. When merged, bands of equal intensity at 23°C and 37°C appear yellow. (B) Signal intensity of each band at 23°C and 37°C was measured, and graphed using ImageJ. Samples at 23°C and 37°C were normalized so that the peak representing Nab2-PrA was equal. 23°C samples are illustrated in red and 37°C in blue. Molecular weight decreases from left to right on the x-axis, and signal intensity is measured on the y-axis.

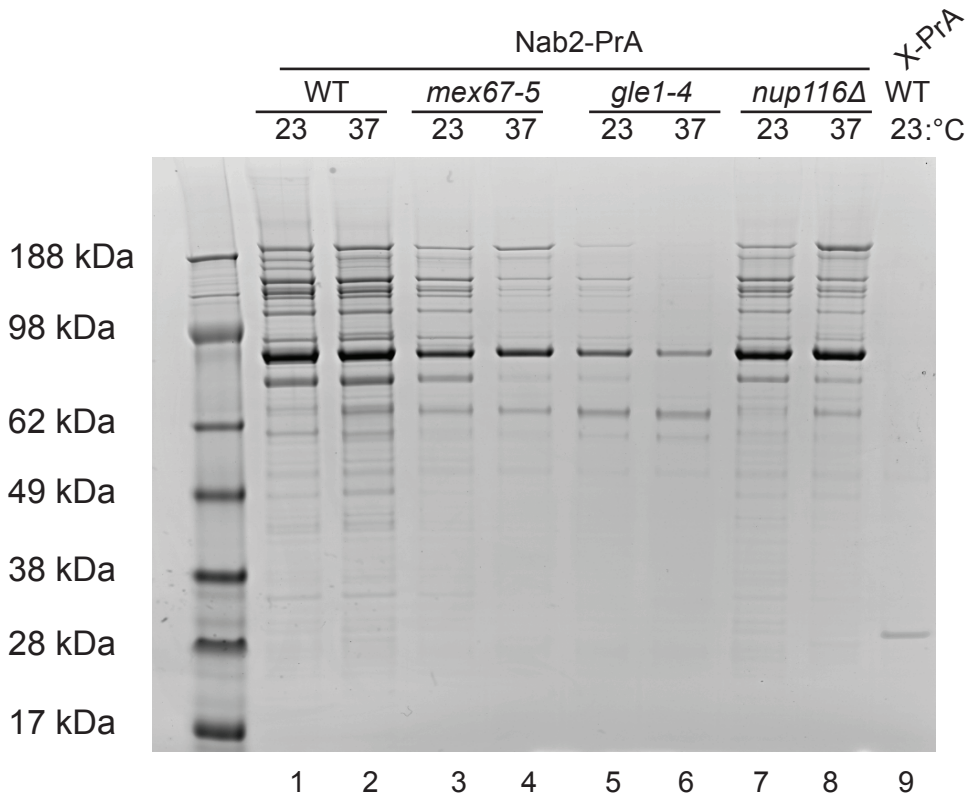


Figure A6. Nab2-PrA complex analysis in wild type, *mex67-5*, *gle1-4*, and *nup116*Δ, from permissive, and non-permissive temperature under medium stringency binding conditions. Strains were grown in YPD at 23°C until late log phase (OD_{600} ~ .8), and one half of culture was harvested. Equal amounts of 65°C YPD were added to remaining culture, and grown at 37°C for 1 hour before harvesting to induce the phenotype of the mRNA export mutants. Purifications were carried out in TBT buffer + 100mM NaCl for 30 minutes at 4°C. RNase A was added to each sample at a final concentration of 100 µg/ml before being added to the beads. Each lane represents 1 gram of cell powder. A purification from a strain harboring PrA not fused to an endogenous protein was included as a negative control (lane 9).

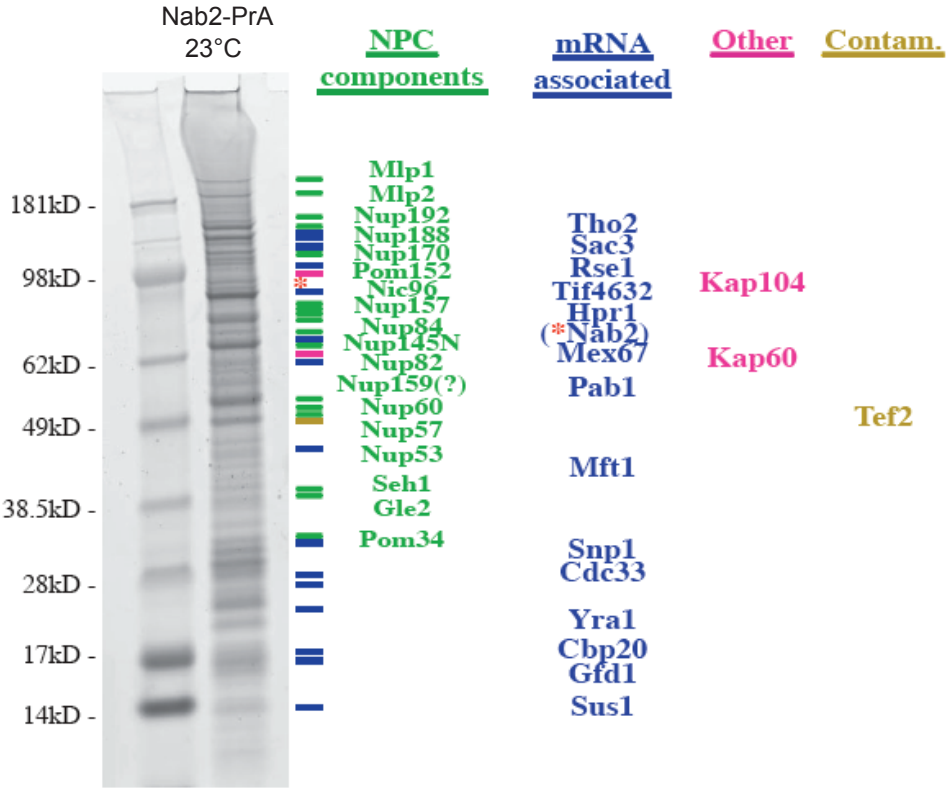


Figure A7. Mass spectrometry analysis of Nab2-PrA purified from cells are 23°C. Nab2-PrA samples were purified from 1 gram of wild type cell powder, from cells grown in YPD at 23°C. Purifications were carried out in TBT + 100mM NaCl, and resolved on a 4-20% acrylamide bis-tris SDS-PAGE gel. Gel bands were excised in 1 mm sections, and subjected to MALDI-TOF mass spectrometry. Identified proteins are ordered as either, 'NPC components', 'mRNA-associated', 'Other', or 'Contaminants'.

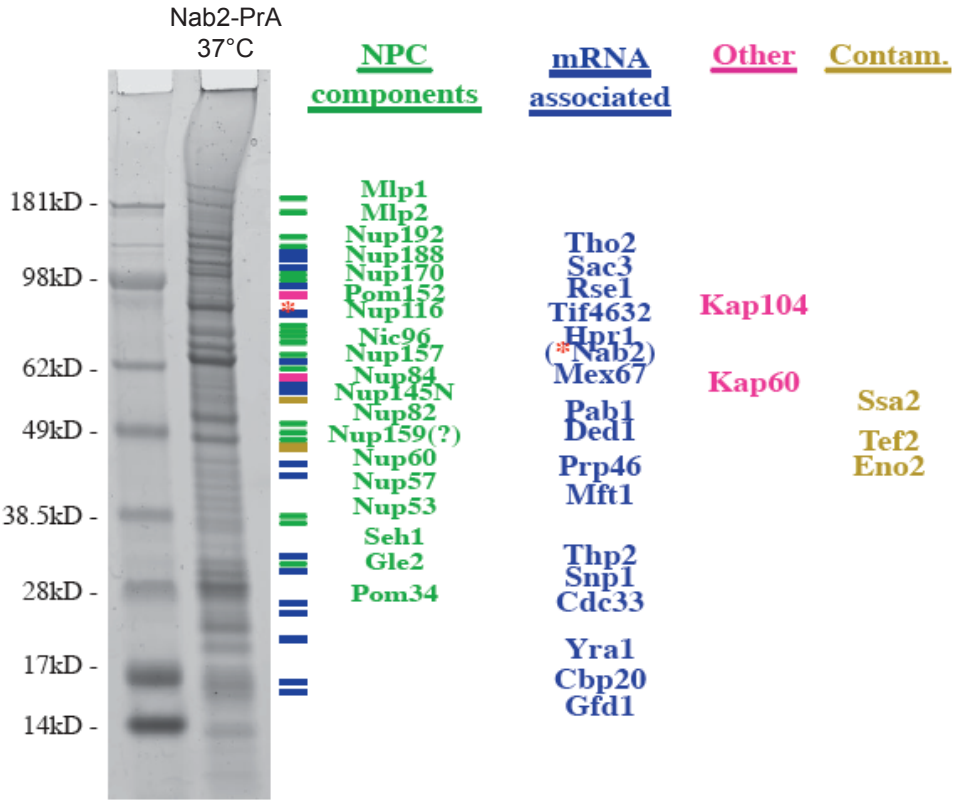


Figure A8. Mass spectrometry analysis of Nab2-PrA purified from cells are 37°C. Nab2-PrA samples were purified from 1 gram of wild type cell powder, from cells grown in YPD at 37°C. Purifications were carried out in TBT + 100mM NaCl, and resolved on a 4-20% acrylamide bis-tris SDS-PAGE gel. Gel bands were excised in 1 mm sections, and subjected to MALDI-TOF mass spectrometry. Identified proteins are ordered as either, 'NPC components', 'mRNA-associated', 'Other', or 'Contaminants'.

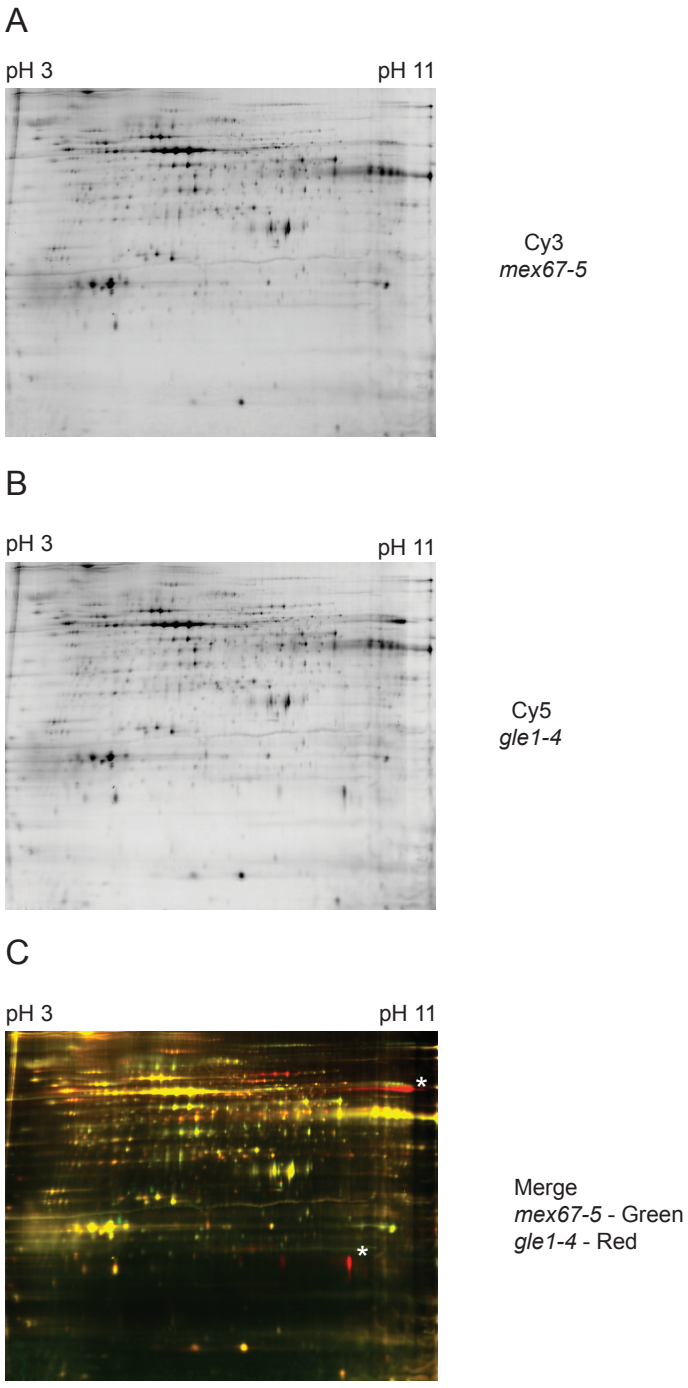


Figure A9. Mex67 and Mtr2 are not present in Nab2-PrA samples purified from *mex67-5* at 37°C. Nab2-PrA was purified from *mex67-5* and *gle1-4* grown at 37°C for 1 hour. The sample purified from *mex67-5* labeled with a Cy3 dye, and the sample from *gle1-4* was labeled with a Cy5 dye. Samples were combined and two dimensional gel electrophoresis was performed. Nab2-PrA from *mex67-5* is seen in (A), Nab2-PrA from *gle1-4* is seen in (B), and the merged samples is in (C), where *mex67-5* sample is green, and the *gle1-4* sample is red. Two proteins were absent in the *mex67-5* sample, Mex67 and Mtr2. These are marked in (C) by white asterisks.

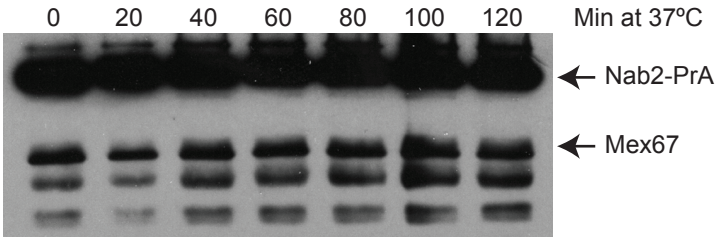


Figure A10. Mex67 protein is stable in *mex67-5* at 37°C. A Nab2-PrA *mex67-5* was grown to mid log phase at 23°C in YPD, and shifted to 37°C for 120 minutes. Aliquots were taken every 20 minutes, and whole cell lysates were prepared. Mex67 stability was analyzed by SDS-PAGE and immunoblotting using a Mex67 antibody. Nab2-PrA is detected due the cross reactivity of the Mex67 antibody and the PrA tag, and serves as a loading control.

B. Analysis of human Dbp5 and Gle1 in stress granules

Observations from Chuck Cole's laboratory showed that yeast Dbp5 localizes to cytoplasmic foci during heat shock in both wild type and *nup42Δ* cells (Scarcelli et. al., 2008). Further, when the human homologue of Nup42, hCG1, is knocked down by siRNA in tissue culture cells, human (h)Gle1 forms cytoplasmic foci (Kendirgi et. al., 2005). Due to these findings, we examined both hDbp5 (Figure B1) and hGle1 (Figure B2) localization during stress conditions known to induce stress granule formation. Strikingly, both hDbp5, and hGle1 localize to stress granules. Of note for future investigation is that hDbp5 localization is much more dramatic that hGle1. EGFP-hDbp5 is found to co-localize with stress granules in nearly 100% transfected cells, while EGFP-hGle1 co-localizes to stress granules at a much lower percentage. The reason for this difference is currently not known.

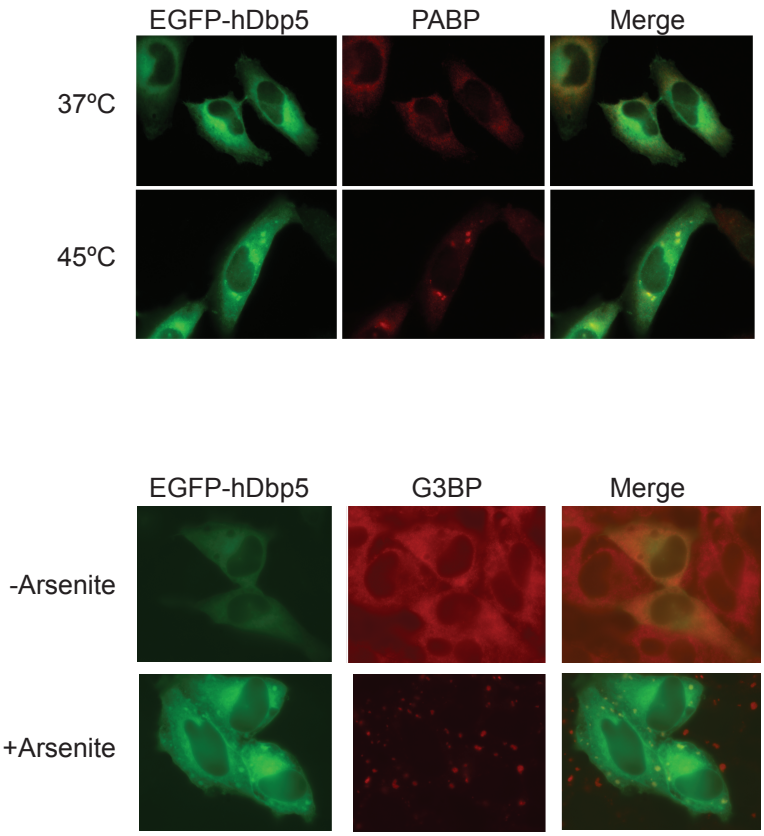


Figure B1. Human Dbp5 localizes to stress granules during cellular stress. (A) EGFP-hDbp5 co-localizes with the stress granule marker PABP in HeLa cells after growth at 45ºC for 1 hour. (B) EGFP-hDbp5 co-localizes with stress granule marker G3BP after 1 hour treatment with 5 mM sodium arsenite.

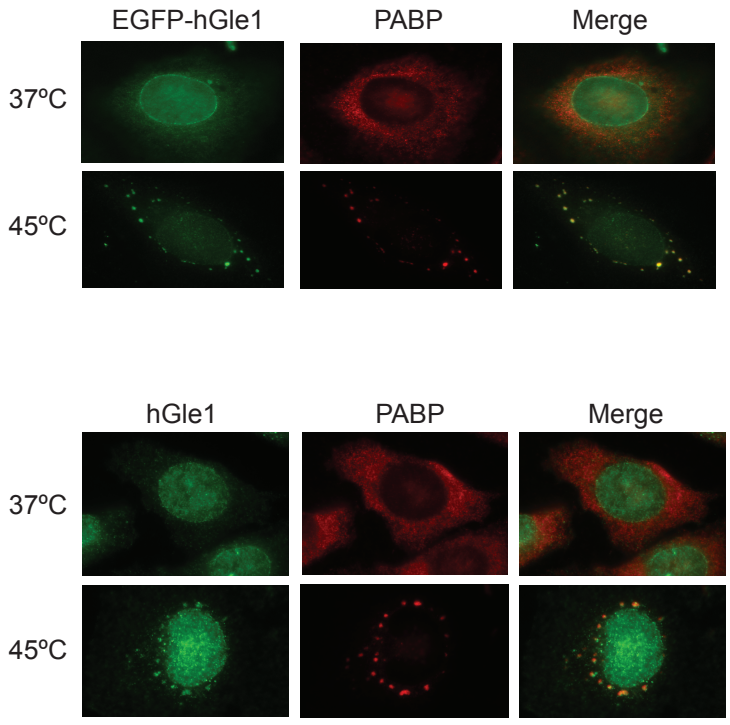


Figure B2. Human Gle1 localizes to stress granules during cellular stress. (A) EGFP-hGle1 co-localizes with the stress granule marker PABP in HeLa cells after growth at 45ºC for 1 hour. (B) Enogenous Gle1 with stress granule marker PABP after growth at 45ºC for 1 hour.

C. Yeast strains used in this study

Strain	Genotype	Source
SWY 3208	Matα *MTW1-CFP:KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3209	Matα *MTW1-YFP:KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3210	Matα *MTW1-CFP:KAN ade2-1 ura 3-1 his 3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup170-1::HIS3*	This study
SWY 3211	Matα *MTW1-YFP:KAN ade2-1 ura 3-1 his 3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup170-1::HIS3*	This study
SWY 3492	Mata *NPL3-TAP:HIS3MX6 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3493	Mata *NAB2-TAP:HIS3MX6 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	
SWY 3494	Mata *NPL3-TAP:HIS3MX6 gle1-4(ts) ade2 ADE3 ura3 trp1*	This study
SWY 3495	Mata *NAB2-TAP:HIS3MX6 gle1-4(ts) ade2 trp1 ura3 leu2*	This study
SWY 3496	Mata *NPL3-TAP:HIS3MX6 nup116-6::URA3 (ts) trp1 leu2 ura3 his3 can1-100*	This study
SWY 3497	Mata *NAB2-TAP:HIS3MX6 nup116-6::URA3 (ts) trp1 leu2 ura3 his3 can1-100*	This study
SWY 3503	Mata *NPL3-TAP:HIS3MX6 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3504	Mata *NAB2-TAP:HIS3MX6 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3505	Matα *nup170-1::HIS3 DCP2-CFP:KAN ade2-1 ura 3-1 his 3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3506	Matα *nup170-1::HIS3 DHH1-CFP:KAN ade2-1 ura 3-1 his 3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3507	Mata *GLE1-YFP:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3508	Mata *NUP159-YFP:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3555	*MEX67::KAN pRS316-MEX67 (MEX67 URA3) ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3591	Mata *NAB2-TAP:HIS3MX6 MEX67::KAN p(mex67-5 TRP1) ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3592	Mata *NPL3-TAP::HIS3MX6 MEX67::KAN p(mex67-5 TRP1) ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3593	Mata *NAB2-TAP:HIS3MX6 MEX67::KAN p(MEX67 URA3) ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3594	Mata *NPL3-TAP:HIS3MX6 MEX67::KAN p(MEX67 URA3) ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3602	Mata *NAB2-TAP-HIS3MX6 RRP6::KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3604	Mata *RRP6::KAN leu2 ura3 his3*	This study
SWY 3605	Matα *NAB2-TAP:HIS3MX6 RRP6::KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3606	Mata *RRP6::KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3607	Matα *RRP6::KAN ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3610	Mata *NAB2-TAP:HIS3MX6 RRP6::KAM gle1-4 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study

SWY 3611	Matα *NAB2-TAP:HIS3MX6 RRP6::KAN gle1-4 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3638	Mata *NAB2-TAP:HIS3MX6 RRP6::KAN nup116::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3639	Matα *NAB2-TAP:HIS3MX6 RRP6::KAN nup116::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3707	*tom1::KAN Stm1-TAPHIS3MX6 leu2 ura3 his3*	This study
SWY 3851	Mata *MEX67::KAN (pmex67-5 TRP1) ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3852	Matα *MEX67::KAN (pmex67-5 TRP1) ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3853	Mata *NAB2-ProteinA:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3870	Matα *NAB2-ProteinA:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3871	Matα *NAB2-Prot:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3872	Mata *NAB2-ProtA:HIS5 mex67::KAN (pmex67-5 TRP1) ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3873	Matα *NAB2-ProtA:HIS5 mex67::KAN (pmex67-5 TRP1) ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3874	Mata *NAB2-ProtA:HIS5 nup116::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3875	Matα *NAB2-ProtA:HIS5 nup116::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3886	Mata *NAB2-ProtA:HIS5 gle1-4 URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 3887	Matα *NAB2-ProtA:HIS5 gle1-4 URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4014	Matα *dbp5-2 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4015	Matα *dbp5-2 ade2-1 ura3-1 HIS3,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4018	Mata *gle1-4 RRP6::KAN Nab2-ProtA:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4019	Mata *GLE1 RRP6::KAN Nab2-ProtA:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4036	Matα *HRB1-ProtA:HIS5 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4087	Mata *nup159-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4088	Matα *nup159-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY 4307	Matα *NAB2::HIS3 ura3 leu2 trp1* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY 4308	Matα *NAB2::HIS3 ura3 leu2 trp1* + pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY 4309	Matα *NAB2::HIS3 ura3 leu2 trp1* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY 4435	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pAC 636 *(NAB2 CEN LEU2)*	This study
SWY 4436	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pSW3579 *(nab2-T178A/S180A CEN LEU2)*	This study
SWY 4437	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY 4438	Mata *NAB2::HIS3 dbp5-2 trp1 leu2* + p(*nab2C437S/CEN/LEU2*)	This study
SWY 4439	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY 4440	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study

SWY 4441	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP1 LYS ADE2 ADE3* + pSW3579 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY 4442	Matα *NAB2::HIS3 gle1-2 ura3 leu2 TRP LYS ADE2 ADE3* p(*nab2C437S/CEN/LEU2*)	This study
SWY 4443	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pAC 636 (*NAB2 CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY 4444	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pSW3579 (*nab2-T178A/S180A CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY 4445	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* + pSW3580 (*nab2-T178E/S180E CEN LEU2*) p(*mex67-5 TRP1*)	This study
SWY 4446	Mata *NAB2::HIS3 trp1 leu2 MEX67::KAN* p(*nab2-C437S*) p(*mex67-5 TRP1*)	This study
SWY 4481	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY 4482	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1*+ pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY 4483	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY 4484	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* p(*nab2C437S/CEN/LEU2*)	This study
SWY 4485	Matα *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY 4486	Matα *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1*+ pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY 4487	Matα *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* + pSW3580 (*nab2-T178E/S180E CEN ALEU2*)	This study
SWY 4488	Matα *NAB2::HIS3 MLP1::KAN ura3 leu2 trp1* p(*nab2C437S/CEN/LEU2*)	This study
SWY 4493	Matα *NAB2::HIS3 NUP42::KAN ura3 leu2 trp1* + pAC 636 (*NAB2 CEN LEU2*)	This study
SWY 4494	Matα *NAB2::HIS3 NUP42::KAN ura3 leu2 trp1*+ pSW3579 (*nab2-T178A/S180A CEN LEU2*)	This study
SWY 4495	Matα *NAB2::HIS3 NUP42::KAN ura3 leu2 trp1* + pSW3580 (*nab2-T178E/S180E CEN LEU2*)	This study
SWY 4496	Matα *NAB2::HIS3 NUP42::KAN ura3 leu2 trp1* p(*nab2C437S/CEN/LEU2*)	This study
SWY 4524	Mata *NAB2::HIS3 GFD1::KAN ura3 leu2* p(*NAB2 CEN URA3*)	This study
SWY 4525	Matα *NAB2::HIS3 NUP42::KAN ura3 leu2* p(*NAB2 CEN URA3*)	This study
SWY 4526	Mata *NAB2::HIS3 MLP1::KAN ura3 leu2* p(*NAB2 CEN URA3*)	This study
SWY 4527	Matα *NAB2::HIS3 MLP1::KAN ura3 leu2* p(*NAB2 CEN URA3*)	This study
SWY 4550	Mata *NAB2-TAP:HIS3MX6 SLT2::KAN leu2 ura3 his3*	This study
SWY 4551	Matα *NAB2-TAP:HIS3MX6 SLT2::KAN leu2 ura3 his3*	This study
SWY 4552	Mata *NAB2-mCherry:HygB YRA1-GFP:HIS5 his3D1 leu2D0 ura3D0*	This study
SWY 4566	Matα *NAB2::HIS3 ura3 leu2 trp1* + p(*nab2 T178D S180D CEN LEU2*)	This study
SWY 4606	Mata *MEX67-GFP:HIS5 SLT2::KAN leu2 his3 ura3*	This study
SWY 4607	Matα *MEX67-GFP:HIS5 SLT2::KAN leu2 his3 ura3*	This study
SWY 4608	Mata *MEX67-GFP:HIS5 MLP1::KAN leu2 his3 ura3*	This study
SWY 4609	Matα *MEX67-GFP:HIS5 MLP1::KAN leu2 his3 ura3*	This study
SWY 4610	Mata *YRA1-GFP:HIS5 SLT2::KAN leu2 his3 ura3*	This study
SWY 4611	Matα *YRA1-GFP:HIS5 SLT2::KAN leu2 his3 ura3*	This study
SWY 4612	Mata *YRA1-GFP:HIS5 MLP1::KAN leu2 his3 ura3*	This study
SWY 4613	Matα *YRA1-GFP:HIS5 MLP1::KAN leu2 his3 ura3*	This study

D. Plasmids used in this study

Plasmid Name	Gene Cloned	Source
pSW 3579	Nab2-T178A/S180A	This study
pSW 3580	Nab2-T178E/S180E	This study
pSW 3610	GST- Nab2-T178E/S180E	This study
pSW 3629	GST- Nab2-T178E/S180E	This study
pSW 3630	GST-Nab2-T178D/S180D	This study
pSW 3631	Nab2-T178D/S180D	This study

E. Methods

Coupling of Dynabeads with Rabbit IgG in order to produce magnetic beads which are capable of pulling out various Protein A tagged complexes. Adapted from Mike Rout lab, Rockefeller University.

Use rabbit IgG -100mg - from Sigma – catalogue number 15006
Dynabeads are from Invitrogen - Dynabeads M270 Epoxy

Day One – Preparing the Beads and Conjugation

1) Resuspend entire vial of Dynabeads (2×10^{10} beads) in 16ml of 0.1M $NaPO_4$ buffer - pH 7.4.

2) Vortex bottle 30 seconds.

3) Divide bead suspension into four 15 ml Falcon tubes (4mL of suspension in each tube).

4) Wash any remaining beads in the glass vial with an additional 2ml of 0.1M $NaPO_4$ buffer. Divide equally amongst the four Falcon tubes.

5) Shake bead suspension slowly for 10 minutes on a Nutator or rocking platform.

6) While bead suspension is on Nutator prepare the AB mix.

   a. FIRST: Resuspend the entire bottle of Rabbit IgG (100mg) in 7ml double distilled H_2O– this will result in a concentration ~14 mg/mL. Aliquot into 1 mL fractions and store any unused IgG at -20°C. Vortex to resuspend. NOTE: After resuspension, solution will often still look somewhat cloudy. This is normal.

   b. Spin down 3525uL, of Rabbit IgG, in a table top centrifuge, for 10 mins, at 14K rpm, at 4°C. Save supernatant and discard pellet. NOTE: Pellet size can vary, from small to medium. This is also normal.

   c. Prepare antibody mix by adding the solutions to a 50mL falcon tube, in the order listed.

      i) 3525uL of IgG (which was previously spun down) *If IgG concentration needs to be altered please see end of protocol for instructions on determining appropriate amounts of Sodium Phosphate and Ammonium Sulfate*

      ii) 9.850mL 0.1M $NaPO_4$ buffer

iii) 6.650mL 3M Ammonium Sulfate. Add slowly shaking the tube a bit.

iv) Filter solution using a .22 µm Millex GP filter

7) Place the Falcon tubes with the bead suspension onto a magnetic holder and wait until all beads are attached to the magnet (Dynal MPC-6 Magnetic Particle Concentrator Prod. No. 120.02). Bead solution will appear clear. Aspirate the buffer off (be careful not to aspirate off the beads too).

8) Wash again with 4mL 0.1M $NaPO_4$ - incubation for 10 minutes is not necessary. Vortex 15 seconds. Put on the Magnet - aspirate off the buffer.

9) Add 5ml of antibody mix to each tube vortex to completely combine the antibody mix and the beads.

10) Wrap tops of Falcon tubes with Parafilm and place on rotating wheel at 30°C over night (incubation must last at least 18 hours but no more than 24 hours).

Day Two - Washing the Dynabeads after Conjugation

Do all washes as described in the 15mL Falcon tubes. You can aspirate the supernatant by using a Vacuum Aspirator.

1) Wash once with 3mL of 100mM Glycine HCL pH2.5. Resuspend and aspirate as fast as possible.

2) Wash once with 3mL of 10mM Tris pH 8.8.

3) Wash once with 3mL of 100mM Triethylamine. (Make fresh 100 mM Triethylamine by adding 168ul stock to 11.15mL of ddH_2O). Resuspend and aspirate as fast as possible.

4) Wash the coated beads with 1x PBS for 5 minutes – washes should be done on a rocker/nutator – repeat 4x.

5) Wash once with PBS + 0.5% Triton X-100 for 5 minutes. Add Triton X-100 fresh before use to PBS.

6) Wash again with PBS +0.5% Triton X-100 for 15 minutes on rocker/nutator.

7) Finally, resuspend all beads in a total of 2ml of 1x PBS + 0.02% Sodium Azide.

8) Store the coated beads at 4 ^{0}C.

Change in IgG volume

Original total reaction volume of the IgG, Sodium Phosphate, and the Ammonium sulfate is 20mL. To determine the new volume of Sodium Phosphate subtract the amount of IgG, and Ammonium sulfate from the original total reaction mixture (20mL). This will leave you with the new volume of Sodium phosphate. *Note: Only two volumes are changing in this reaction; the volume of IgG and the volume of Sodium Phosphate. The among of Ammonium Sulfate remains constant.*

Necessary Solutions

0.1M Sodium Phosphate Buffer ($NaPO_4$) – pH 7.4

2.62g NaH_2PO_4 x H_2O (MW 137.99)
14.42g Na_2HPO_4 x $2H_2O$ (MW 177.99)

Dissolve in ddH_2O, adjust pH if necessary and adjust to 1 liter.

3M Ammonium Sulfate (stock solution)

39.6g $(NH_4)_2SO_4$ (MW 132.1)

Dissolve in 0.1M Sodium Phosphate Buffer (pH 7.4) and adjust to 100mL

Phosphate Buffered Saline (PBS) - pH 7.4

0.26g NaH_2PO_4 x H_2O (MW 137.99)
1.44g Na_2HPO_4 x $2H_2O$ (MW 177.99)
8.78g NaCl (MW 58.5)

Dissolve in 900mL distilled water, adjust pH if necessary and adjust to 1 liter.

PBS + 0.5% Triton X-100

Include 0.5% (w/v) Triton X-100 in 100 mL PBS solution

100mM Glycine HCl pH 2.5
10mM Tris pH 8.8
10% Sodium Azide (NaN_3)

NOTE: I filter all solutions, and only use disposable pipettes for the conjugation protocol because any keratin, etc, that is in glassware, pipettes, etc, can be conjugated to the beads.

Harvesting Cells and Making Noodles

This protocol is designed to harvest a yeast cell culture and prepare it for grinding.

1. Grow cell culture to at least $3.0x10^7$ cell/mL total.

2. Spin cultures down at 4000xg, for 10 minutes, 4°C

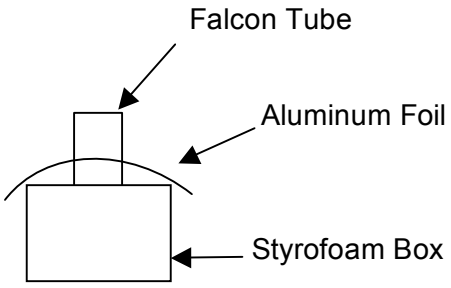
3. Wash cell pellet by resuspending pellet with 50mL ddH_2O over ice. Put resuspended solution into 50mL Falcon tube(s) and spin down at 2600xg, for 5 minutes, at 4°C. Repeat this 1x.

4. Resuspend pellet, over ice, with a volume of resuspension buffer equal to the volume of the pellet. Spin down at 2600xg, for 15 minutes. Aspirate all liquid from the pellet.

5. Spin down again (just the pellet), at 2600xg for 15 minutes to ensure all of the buffer is removed. (This step is not necessary.)

6. Pellet should be fairly dry and resemble a thick paste.

7. Place liquid nitrogen in a styrofoam container, top with aluminum foil and place a 50mL Falcon tube through a hole in the foil. Allow tube to cool. (See picture below.)



8. Fill the cooled 50mL falcon tube, to the very top, with liquid nitrogen.

9. With a spatula, scoop out cell paste and place into a 10mL or 20mL syringe. Press out the cell paste into the liquid nitrogen in the Falcon tube.

10. When all cell paste is gone, decant liquid nitrogen from the tube (try not to lose any noodles, can pour off liquid nitrogen by poking holes into cap of Falcon tube, screw on the cap and then turn tube upside down to pour out the liquid nitrogen.).

11. Do not tighten the tube completely, in order to allow liquid nitrogen vapor to escape. Store tubes at -70-80°C.

Resuspension Buffer:
1.2% PVP-40
20 mM Hepes pH 7.4

Note: Before using the resuspension buffer, add the following solutions to the volume of buffer you intend to use.

1:100 PIC (Protease inhibitor tablet. This is no longer used.)

1:100 Solution P (Sol. P= 2mg pepstatin A, 90 mg PMSF in 5 ml 100%EtOH. Store at -20C, discard after 3 weeks.)

1:1000 of DTT. (1mM final)

Cryogenic Disruption of Yeast (Retsch PM 100) - Adapted from Mike Rout lab, Rockefeller University.

Lysing of frozen yeast cells using a planetary ball mill.

1) Always wear gloves, and protect hands.

2) Fill a rectangular ice bucket about ¼ full with liquid nitrogen.

3) Pre-chill everything. Immerse the stainless steel grinding jars, the stainless steel lid, the grinding balls and the storage tube, with the frozen yeast noodles, in the liquid nitrogen.

4) Pre-cooling is finished when nitrogen bath is no longer bubbling vigorously.

5) Once everything is chilled pour the noodles into the 125 mL grinding jar.

6) Make sure the counterbalance is correct.

7) Using the 125 ml jar, use 11 of the 20mm stainless steel balls.

8) Be sure ***no liquid nitrogen*** is in the grinding jar prior to grinding to avoid an explosion.

9) Grinding is done in 8 cycles, each cycle is set in the following manner:
   - 400 RPM
   - 3 minutes
   - 1 minute reverse rotation with no breaks between rotations

   (*NOTE*: You MUST hear the balls rattling around in the jar! If there is no rattling then add/remove balls to the jar until you hear it rattle. It is not considered a grinding cycle unless there is rattling.)

10) Between each cycle the jars are removed and cooled in liquid nitrogen. Do not remove the lid (removal of lid may result in cell loss)! To ensure lid is chilled use an empty Falcon tube to pour liquid nitrogen over the top of the grinding jar while the bowl of the grinding jar cools in the liquid nitrogen bath. DO NOT SUBMERGE THE JAR COMPLETELY as this will allow liquid nitrogen into the grinding bowl and may also result in cell loss, or explosion of jar when sealed.

11) When 8 cycles are complete remove powder with a spatula – There is often powder stuck on the side of the jar. Try to loosen with spatula. If it is too hard to remove, repeat 1 grinding cycle at 350 RPM, 2 minutes, 1 minute reverse rotation no breaks between rotations.

12) Jars and balls can be with warm soapy water. Rinse very well with ddH_2O, so no soap remains for next use.

13) Typically ~90% of yeast cells can be disrupted in such procedure. Frozen ground cells are stored at -70-80ºC.

Pullouts Using Dynabeads and Cell Powder Filtration

From M. Rout lab, adapted by S. Carmody

1. Prewash Dynabeads 3x in 1ml of extraction buffer. Resuspend in volume equal to initial volume taken, and place in 15ml conical tube on ice

2. Weigh out desired amount of cell powder, and thaw briefly on ice until partially thawed (should look a little thicker than soft serve ice cream)

3. Resuspend in extraction buffer and vortex and 45-60 seconds, until in solution

    - For every .5g of cell powder, resuspend in 4.5ml of extraction buffer and bind to 25µl of dynabeads. Scale up appropriately as needed.

4. Spin in Jouan 2.4k rpm, 2 min, 4ºC

5. Carefully pour off supernatant into a 20ml syringe, being careful to avoid pellet

6. Clear air from syringe, and filter through Whatman 25mm GD/X filter (1.6µm pore size, Cat. No. 6882-2516) directly into 15ml conical containing beads. Incubation time starts now

7. Bind 30 minutes rotating/nutating at 4ºC

8. Collect on 15ml conical magnet, and resuspend in 1ml extraction buffer. Move to Eppendorf tube, and wash 3x 1ml extraction buffer

9. Wash 1x with last wash buffer (made fresh), nutating for 5 minutes at RT
    - IF RUNNING A GEL WITH SAMPLES PROCEDE TO STEP 10, IF SENDING FOR LC/MS ANALYSIS, WASH 3X WITH LW BUFFER WITHOUT DETERGENT, NUTATING THE LAST WASH 5 MINUTES AT RT

10. Elute 2x with 500µl elution buffer, nutating each 20 minutes at RT, and pooling elutions

11. Poke hole in top of tube, and speed vac dry ON. Do not heat speed vac, it is not necessary

12. In the morning, collect samples, and if running a gel with them, resuspend in 8µl solution A, if sending for LC/MS analysis, keep as dried pellet

13. Heat 5 min at 70ºC

14. Spin 2 min, 13k rpm, RT

15. Put on magnet, and remove protein sample from residual dynabeads that may be in tube

16. Add 8 µl solution B, flick to mix

17. Heat 70ºC 10 min

18. Spin 2 min 13k rpm RT

19. If staining gel with coomassie, run all of sample. If western blotting make 1:100 dilution of sample and load ~2-20µl as a safe starting point. This can be variable, and may require more or less sample depending on the target and interacting protein

Buffers:

Last Wash Buffer (Make fresh each time):
333µl 3M NH_4OAc pH = 7.5
1µl 2M $MgCl_2$
4µl 50% Tween-20 (Can use 2µl 100%, but 50% goes into solution easier)
Up to 10ml with ddH_20
(For LW for LC/MS omit Tween-20)

Elution Buffer (Make fresh each time):
338µl NH_4OH from stock (.5M final concentration)
10µl .5M EDTA (.5mM final concentration)
Up to 10ml total with ddH_2O

Solution A:
.5M Tris pH = 8.0
5% SDS
(Store at room temp)

Solution B:
37.5ml glycerol
12.5ml ddH_2O
.96g DTT (124.5mM final concentration)
.05% BPB
(Dissolve DTT, and BPB in H_2O, then add glycerol. Store at -20ºC)

Solution P:
90mg PMSF
2mg Pepstatin A
5ml 100% EtOH
(Vortex well and nutate, Pepstatin does not go into solution easily)

TBT Buffer (A typical extraction buffer used by Rout lab, and now us)
20 mM HEPES pH=7.4
110 mM KOAc
.1% Tween
2 mM $MgCl_2$
(NaCl can be omitted or added up to 1M depending on target protein, and desired stringency of purification)
I make the above in a 10x stock, and add the below fresh before each use
.5% Triton X-100 (250µl/50ml buffer)
250µl Solution P/5ml buffer
3µl Antifoam B (shake well before use)/50ml buffer

FRENCH PRESS LYSIS PROTOCOL

(Follow steps 1-6 if using suction method to load sample)

1) Grease all O-rings (3), making sure that they are not too greasy. Vacuum grease should be coat the rings, but not be thick.

2) Put cell onto the base, screw side up

3) Push plunger into cell, with the "STOP" and "MAX FILL" facing forward

4) Put cell onto the press, making sure that the surface of the press is flat (no debris on it) and tighten screws to hold down the cell

5) Screw in outflow tube and release valve. Do not screw release valve too tight, nor too loose. (If valve is too tighten, suction will be difficult. If valve is too loose, you will suck in air.)

6) Suck up sample by lifting plunger. Stop once all sample is in the cell, taking care not to get air in sample. (Air=frothy; sample=bad). Keep conical tube on ice, so that sample exits into a cold tube.

7) Tighten release valve just past finger tight. DO NOT OVERTIGHTEN THE VALVE.

8) Turn on machine. Turn ratio selector to "Medium," and "Pressure Increase" to 500 on the meter. The press will rise, resulting in a pressure increase within the cell. Keep outflow tube in hand, and unsure that it does not leak when pressure is applied. If leakage occurs, rapidly but gently tighten valve until leakage stops. Again, DO NOT OVERTIGHTEN THE VALVE.

9) Bring ratio selector up to "high" and "Pressure Increase" to "1000." At both steps, make sure that there is no leakage and tighten valve accordingly if there is. Again, DO NOT OVERTIGHTEN THE VALVE. "1000" on high ~16k psi.

10) Now that there is no leakage, open the valve *slowly*, so that your sample comes out in a dropwise fashion. If sample is flowing out, tighten column gently until it comes out dropwise. Sample will periodically stop flowing. Slightly open the valve again for dropwise flow.

11) When the "STOP" on the plunger begins to be obscured, tighten valve so that flow stops. DO NOT OVERTIGHTEN THE VALVE, if flow does not stop with slight tightening, move quickly, but carefully to #12.

12) Lower pressure to “500” on the meter. Lower ratio selector to “MED.” Lower pressure to 0, moving knob completely to the left. Lower ratio selector to “down.” Press will now come down.

13) Open valve to allow for dead volume sample to come out.

a

14) Repeat for as many passes as desired (2 is usually sufficient.)

Bis-tris SDS-PAGE Preparations

Bis-tris gels tend to give sharper banding patterns, and as such can be used instead of tris glycine gels in cases where resolution of numerous bands is desired (eg, in TAP-tag isolations.)

Resolving Gel Recipe (8% acrylamide)

1 gel	2 gels
1.42 ml 1.25M Bis-tris pH=6.8	2.84 ml 1.25M Bis-tris pH=6.8
1.25 ml 32% acrylamide (15:1)	2.50 ml 32% acrylamide (15:1)
2.33 ml ddH_2O	4.66 ml ddH_2O
33 µl 1M APS	66 µl 1M APS
9.3 µl TEMED	18.6 µl TEMED

Stacking

1 gel	2 gels
1 ml 1.25M Bis-tris pH=6.8	2 ml Buffer 1.25M Bis-tris pH=6.8
437 µl 32% acrylamide (15:1)	874 µl 32% acrylamide (15:1)
2.1 ml ddH_2O	4.2 ml ddH_2O
23 µl 1M APS	46 µl 1M APS
6.5 µl TEMED	13 µl TEMED

Running Buffer

Run gels in 1x MES running buffer + 5mM sodium bisulfite (1M stock diluted 1:200) (20x stock- MES SDS-PAGE running buffer, NuPage Cat. #NP0002)

*Sodium bilsulfite used is from Fisher (S654-500). It is a mixture of sodium bisulfite, and sodium metabisulfite (a compound which dissociates into 2 sodium bisulfite molecules in H_2O.) Being a mixture, there is no formula weight listed on the bottle. To calculate the formula weight divide 19211.56 by the assay percent listed on the bottle (eg, 66.6%). Divide this number by 3, and that is the effective formula weight.

eg, 19211.56/66.5 = 288.90, 288.90/3 = 96.29 -> 96.29 g/L = 1M

Staining/Transferring gels

After running, gels can be treated identically to tris glycine gels, and stained or transferred as desired.

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